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This report describes the work conducted in an expiring 2y IDEA for which a no cost extension was approved. Our original proposal was funded to study a human estrogen regulated transcript, HEM45. Recent homology studies indicate that HEM45 is related to a family of proteins that have an exonuclease domain. These include DNA proof-reading proteins and proteins associated with mitotic structures.

Studies in the project have demonstrated altering patterns of HEM45 distribution detectable in the cell cycle and an association with the mitotic spindle. These data support a role for HEM45 in the cell cycle/proliferation, however we have not been able to significantly perturb levels of native HEM45 transfected fusion protein in cells and cell extracts.

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5. INTRODUCTION:

This report describes the work conducted in an expiring 2y IDEA for which a no cost extension was approved. Our original proposal was funded to study a human estrogen regulated transcript, HEM45. Recent homology studies indicate that HEM45 is related to a family of proteins that have an exonuclease domain. These include DNA proof-reading proteins and proteins associated with mitotic structures.
Studies in the project have demonstrated altering patterns of HEM45 distribution detectable in the cell cycle and an association with the mitotic spindle. These data support a role for HEM45 in the cell cycle/proliferation, however we have not been able to significantly perturb levels of native HEM45 transfected fusion protein in cells and cell extracts.

6. BODY:

This section summarizes studies that are principally documented in the journal articles included in the appendices (section 11). In a paper published with the support of this IDEA (1) (appendix item 1) we described studies underlying the grant. Human HEM45 was identified as a differential display product in HeLa cells carrying transfected estrogen receptor. HEM45 was shown to be estrogen regulated in other ER positive cells and a rat analog was found to be estrogen regulated in the Uterus. HEM45 was identified as related to a gene regulating fruiting body formation in fungi, and to a Xenopus gene that can rescue S Pombe from a mitotic catastrophe phenotype, suggesting a cell cycle or growth control function for HEM45. A recent study (2) used informatics tools to identify proteins containing the motif responsible for the proof reading exonuclease activity of E.coli DNA polymerase I. HEM45 was identified as a potential gene encoding an exonuclease motif of this type (see appendix 2 for excerpted alignment), and was placed in a subgroup along with several of the 'neighbors' we had previously identified. This motif is present in several cell cycle control point including the OP18 leukemic factor involved in the dynamics of tubulin polymerisation/depolmerisation (3), and DNA repair exonuclease Rec1.

In addressing intra-cellular localization of HEM45 (our aim 2) as a means to addressing its cellular function/role, we raised antisera to HEM45 using the whole recombinant protein and peptide fragments. The antisera were used to study HEM45 localization, this encompassed tasks 8-13 of the project and resulted in submission of a manuscript (appendix 3) which is in revision.
The HEM45 antisera recognized an appropriately sized (20.3kDa) protein in cell extracts and was used in fluorescence microscopy studies. By microscopy, the localization of HEM45 changes during the cell cycle, with a striking association with the mitotic spindle in metaphase and anaphase. Note that the intensity of HEm45 staining apparently changes from interphase to mitosis. This is largely due to changing sub-cellular associations, with the discrete association with the mitotic spindle making protein more visible. The draft manuscript also noted that by western the level of protein in westerns was increased. This was reported as three fold, but the PI now considers this to likely be an overestimate.

A second group has characterized the HEM45 mRNA as interferon inducible in Daudi cells (4). Note that we have a conflict with that group over subcellular localization of protein, they found a flagged peptide, using transfected chimeric constructs, in the nuclear PML bodies, we found principally cytoplasmic localization. We believe their fixation methods may have flushed the majority of HEM45 from the cytoplasm, prior to analysis, leaving only material associating with the nuclear bodies. Note that our approach used antibody to the HEM45 protein and probed for the endogenous protein , rather than a tag attached to over expressed, transfected protein.

A negative aspects to the project has been a failure affect cell behavior by modifying HEM45 expression. We also tried to rephrase this part of our studies by attempting to affect the cell levels of HEM45 and fusions. In the last report we indicated the difficulty in observing a changed phenotype in response to cell transfection with HEM45 constructs, *i.e.* that the final studies of our original aim1, tasks 6 and 7, were not successful. Our report emphasized the biochemistry of HEM45 as the focus of the next year. A smaller part of our future plans was also HEM45 protein expression by perturbing cell cycle progression with chemical agents. The latter part of the project became the center point of studies as the response from grant and manuscript reviewers was 'why study HEM45, what is its relevance'. Unfortunately we are still unable to perturb HEM45 expression.

Compounds evaluated for cell cycle perturbation of HEM45 included: campothecin, cis-platin, gamma-radiation, 4-NitroloquinolineN-Oxide, Paclitaxel, demi-colcemid Vincristine, Ethidium Bromide, Iodoacetamide, Hydrogen Peroxide, and Hydroxy Urea. This is a fairly comprehensive set of cell-damaging agent, with agent causing both small and large DNA adducts that may be excised by differing mechanism, in addition to intercalating drugs and drugs that perturb the mitotic spindle.
HeLa and HeLa-derived cells were treated for up to 24h or harvested up to 24 h after treatment with drug/radiation. We established a protocol where we would initially determine levels of agent causing cell cycle arrest without massive cell death and then analyze cells under a limited range of selected doses/duration at and below the arresting level. Cell cycle staging was determined by flow cytometry of propidium iodide stained ethanol-fixed cells. Cell analysis for HEM45 expression was either by western blot, studying the endogenous gene, or used flow cytometry to analyze HEM45-GFP fusion protein expression and relationship between HEM45-GFP fusion expression and cell cycle stage (using '2-color Flow' for GFP and propidium iodide). In the latter case we were presuming that the cell would regulate the level of protein in an 'appropriate fashion', a somewhat risky strategy, but justified by the speed and ease of the assay. Neither results for the studies on endogenous HEM45 nor the HEM45-GFP fusion gave convincing data indicting perturbation of HEM45 expression as a result of drug treatment and cell cycle perturbation. Any changes were marginal considering the drastic perturbations of cell cycle progression that we could achieve and monitor following drug/radiation treatments. This data indicates that our hypothesis that HEM45 is involved in cell cycle control is incorrect, or that our tumor cell model is inappropriate for the study. Note that the 3 fold changes we reported in the last figure of the draft manuscript are also very limited when one considers the degree of enrichment for detached, mitotic, cells . We discussed the role of HEM45 Dr Mian, who published the study (2) on the presence of a potential exonuclease domain in HEM45, using informatics tools. She felt that HEM45's closest relatives were more involved in mRNA processing than repair. This could be addressed by biochemical studies with the over expressed protein, however, we are having problems in justification of further study of HEM45 without a clear link to a disease state (whether breast cancer or some other problem).

7. KEY RESEARCH ACCOMPLISHMENTS:

The studies have demonstrated changing patterns of distribution of HEM45 in the cell cycle, with an association with the mitotic spindle. This provides a cell biology link with the recently published observation that HEM45 is part of a group of proteins that have an exonuclease domain and include several cell cycle check-point proteins.

We found that it was not possible to manipulate HEM45 protein expression in cells by transfection methodologies or cell cycle arrest. This may have implications for role of HEM45, or reflect its aberrant control in cultured cells but has the practical effect of making difficult the probing of its action by cell and molecular biology methods.

8. REPORTABLE OUTCOMES:

Developed antisera to HEM45 that are available for our own studies and can be provided to wider scientific community.

Developed cells having Doxycycline-regulated expression systems that may be useful in other studies Visualized HEM45 in cells.

Published one paper and submitted one additional manuscript.

Applied for two research grants (by PI) relating to HEM45 during funding period (see appendix 4) and one national research service award (Post-Doc).

9. CONCLUSIONS:

Th project has been successful in visualizing HEM45 in cells using antisera developed as part of the project and using GFP-HEM45 fusions for limited studies. The project was less successful in perturbing HEM45 expression and detecting effects on cell behavior. A basic question is whether the inability to perturb HEM45 expression indicates a simple technical failure on our part, or a fundamental aspect of HEM45 protein expression, as opposed to the transcription/ mRNA level that are the genral route to altering protein expression. The

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11. APPENDICES:

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APPENDIX 1

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Expression and Estrogen Regulation of the HEM45 mRNA in Human Tumor Lines and in the Rat Uterus

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A human estrogen regulated transcript, HEM45, was characterized that encodes a novel protein of 181 amino-acid residues (M_r 20 300). It was identified using differential-display-PCR and mRNA from a human cervical cancer cell line (UP1) stably transfected with an estrogen receptor (ER) expression construct. The HEM45 protein has similarity to the bracket fungus protein FRT1 that can cause fruiting-body production and to a *Xenopus* product, XPMC2, that affects cell-cycle control. These similarities suggest that HEM45 will have a role in mediating estrogen control of cellular proliferation and differentiation. HEM45 mRNA was widely expressed at low levels in cell lines and was up regulated by E_2 in ER-positive breast cancer lines. The *in vivo* regulation of HEM45 was confirmed by demonstrating estrogen stimulation of the rat HEM45 homolog in the rat uterus. The levels of the rat uterine HEM45 sequence were elevated by estrogen 3 to 15 h after treatment. The maximal response, at six hours, was greater than eight-fold. The uterine HEM45 response was distinct from that reported for 'early-response' genes as the increase in HEM45 mRNA levels occurred later but could be induced by lower levels of hormone. HEM45 mRNA expression in cultured cells was increased by estrogen in the presence of cycloheximide, indicating direct ER-regulation of HEM45. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Interplay of estrogens and progestins is essential in establishing and maintaining pregnancies at the level of the uterus and beyond [1]. Estrogen action is principally mediated by nuclear estrogen receptor (ER), ligand activated transcription factors [2] that transduce the hormonal signal [3]. ER interacts with well-defined DNA binding sites [4] to directly influence transcription of specific genes and to thereby indirectly affect a multitude of pathways and processes. Estrogens are important in both normal uterine development and in pathological uterine conditions such as endometriosis and endometrial cancer. In tumors of the mammary epithelium intact sex steroid response pathways are an indicator of good prognosis [5], and there is growing evidence for the role of estrogen in bone homeostasis in both sexes [6].

In the current study the hypothesis was tested that human cervical cancer cells would provide a relevant model for estrogen action in the reproductive tract, leading to identification of estrogen regulated sequences whose control could then be studied *in vivo* in a rodent model. The initial characterization of sequences in the human cells maximized potential relevance of identified gene products to human endocrine regulation.

Cultured cells offer the most accessible source of human RNA owing to the limited availability of human tissues and the general difficulty of manipulating human hormonal status for research purposes.

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Abbreviations: aa, Amino-acid residue; Bp, Base-pair; CAT, chloramphenicol acetyl transferase; CKB, creatine kinase B; DDPCR, differential display PCR; DCS, donor calf serum; DMEM, Dulbecco's modified Eagle medium; ER, estrogen receptor; ERE, estrogen response element; EST, expressed sequence tag; E_2, 17β-estradiol; IACUC, Institutional Animal Use Committee; IP, intra-peritoneal; MMLV, mouse mammary leukemia virus; Nt, nucleotide; ORF, open reading frame; PCR, polymerase chain reaction; RNA, ribonucleic acid; RPA, RNase protection assay; RT, reverse transcriptase; SEM, standard error of the mean; TIGR, The Institute for Genomic Research.

Cultured human cells have been useful in characterizing gene-products relevant to growth of estrogen-responsive breast cancers and for identifying potential breast cancer tumor markers [5].

A rodent model was used for *in vivo* studies given the limited availability of human tissue with defined hormonal status. The immature rodent uterus has provided a model system for study of estrogen action *in vivo*. A wave of DNA synthesis and cell replication occurs 24–30 h after estrogen administration [7] as a result of the pleiotropic actions of the hormone.

This report describes identification and isolation of a cDNA fragment, termed HEM45, by the profiling of control and estrogenized RNA samples from both ER-positive and ER-negative HeLa-derived cells. HEM45 mRNA is induced by estrogen in all examined cell lines expressing functional ER. In addition, HEM45 mRNA is widely expressed at low basal levels. Sequence analysis indicates that the human HEM45 gene may be related to other genes with tentatively defined cell-regulatory functions.

Preliminary studies on the rat homolog in the immature uterus extend the cell studies to an *in vivo* model and suggest that HEM45 may be generally relevant to mammalian reproductive-tract biology.

METHODS

Creation of ER-positive tumor cell lines by ER transfection

HeLa cells [8] were transfected on 35 mm diameter dishes by the calcium phosphate procedure using a 20:1 mass ratio (10 to 0.5 μg) of the human ER-α expression construct pHE0 [4] to selection vector pUR (Clontech, Palo Alto, CA) [9]. The pUR vector carries a puromycin acetylase gene allowing selection with puromycin. This led to the creation of ER-positive lines BD5 and UP1. Several puromycin-resistant control lines (including CL1, CL2, and CL4) were developed by transfection with pUR alone. Resistant cells were initially selected using puromycin (Clontech, Palo Alto) at 1 μg/ml. ER-positive BD5 and ER-negative control lines CL1, CL2, and CL4 were cloned under this level of selection. Cloned lines were maintained in 1 μg/ml puromycin. ER-positive UP1 was developed by additional selection of mass cultures with 6 μg/ml puromycin. The ER content of whole cell extracts of UP1 cells was 65.7 ± 6.8 fmol/mg protein ($\pm$SEM) by the Abbott (Chicago, IL) ER Enzymic Immuno-assay kit, as compared to 91.7 ± 1.4 fmol/mg protein for MCF7 cell extracts in parallel assays. ER protein in UP1 cells was shown to be functional by transient transfection of UP1 with a TK-CAT expression construct carrying the weak CKB ERE [10]. An eight-fold increase in CAT activity was detected in extracts of cells treated with E_2 for 24 h, as compared to vehicle-treated cells (data not shown).

An ER-positive variant, MDAL3, of ER-negative MDA-MB-231 breast-cancer cells was also developed by stable co-transfection with pHEO and pUR.

Differential display PCR

Differential display PCR-based analysis was by the approach of Liang and Pardee [11, 12] except that α-^{33}P dATP was used as the radio-labeled nucleotide. The differential display survey that identified the HEM45 sequence utilized the dT primer 5′ T_{12}[C&G&A]G 3′ and arbitrary primer 5′GGAGTGCCTC3′ (OPP13, Operon Technologies, Alameda, CA). Re-amplified PCR fragments were ligated to T-tailed, linearized pCRII vector (Invitrogen, San Diego) and competent INVαF′ *E. coli* (Invitrogen) were transformed to ampicillin resistance.

RNAs for DDPCR were from confluent, untreated, ER-positive UP1 cells and the control CL1 line that is puromycin-resistant but ER-negative, and from cultures of both lines exposed to 10 nM E_2 for five days after confluence. RNA was prepared by the centrifugation of cell lysates in guanidinium thiocyanate layered on cesium chloride gradients [13, 14].

RNAse protection assay

RNA probes were prepared and RPAs performed as described [12]. Ten micrograms of total cellular RNA were used in the hybridization step of the RPA protocol. An 82-nt HEM45-protected fragment was generated in RPAs with anti-sense probe synthesized from an HEM45 fragment (Bases 439-521 in Fig. 1) subcloned in pBSK. Radiolabeled HEM45 anti-sense RNA probes were gel-isolated before use in the RPA in order to reduce background, as the levels of the HEM45 mRNA are low.

A 200-bp fragment of the constitutively expressed human 36B4 reference probe [16] (acidic ribosomal phosphoprotein P0) subcloned in pBSK was used as a control with human-derived RNA samples. An invariant differential display product (HINV53) was also used as a reference probe with human RNA samples in some early experiments. The antisense 1A reference probe for rat uterine RNA was generated from a pBSK construct containing a 175-bp *Hin*dIII–*Eco*RI fragment of p1A [17] (subunit 1 of mitochondrial cytochrome oxidase). Radiolabelled antisense reference probes were not gel-isolated before use in the RPA. The rat CKB mRNA protection probe was generated from a pGEM3 construct containing a 140-bp *Hpa*II–*Eco*RI fragment from the 3′ end of the described rat CKB cDNA [13].

The data in RPA gels were collected using a Fuji-Bas 2000 Phosphor Imager (Fuji, Minamiashigara City) or XAR-5 film (Kodak, Rochester, NY). HEM45 levels were normalized, where indicated, to

```
   1  CAGAGGCAGGCAGCATCTCTGAGGGTCCCCAAGGAACATGGCTGGGAGCCGTGAGGTGGT   60
                                              M  A  G  S  R  E  V  V

  61  GGCCATGGACTGCGAGATGGTGGGGCTGGGGCCCCACCGGGAGAGTGGCCTGGCTCGTTG  120
      A  M  D  C  E  M  V  G  L  G  P  H  R  E  S  G  L  A  R  C

 121  CAGCCTCGTGAACGTCCACGGTGCTGTGCTGTACGACAAGTTCATCCGGCCTGAGGGAGA  180
      S  L  V  N  V  H  G  A  V  L  Y  D  K  F  I  R  P  E  G  E

 181  GATCACCGATTACAGAACCCGGGTCAGCGGGGTCACCCCTCAGCACATGGTGGGGGCCAC  240
      I  T  D  Y  R  T  R  V  S  G  V  T  P  Q  H  M  V  G  A  T

 241  ACCATTTGCCGTGGCCAGGCTGGAGATCCTGCAGCTCCTGAAAGGCAAGCTGGTGGTGGG  300
      P  F  A  V  A  R  L  E  I  L  Q  L  L  K  G  K  L  V  V  G

 301  TCATGACCTGAAGCACGACTTCCAGGCACTGAAAGAGGACATGAGCGGCTACACAATCTA  360
      H  D  L  K  H  D  F  Q  A  L  K  E  D  M  S  G  Y  T  I  Y

 361  CGACACGTCCACTGACAGGCTGTTGTGGCGTGAGGCCAAGCTGGACCACTGCAGGCGTGT  420
      D  T  S  T  D  R  L  L  W  R  E  A  K  L  D  H  C  R  R  V

 421  CTCCCTGCGGGTGCTGAGTGAGCGCCTCCTACACAAGAGCATCCAGAACAGCCTGCTTGG  480
      S  L  R  V  L  S  E  R  L  L  H  K  S  I  Q  N  S  L  L  G

 481  ACACAGCTCGGTGGAAGATGCGAGGGCAACGATGGAGCTCTATCAAATCTCCCAGAGAAT  540
      H  S  S  V  E  D  A  R  A  T  M  E  L  Y  Q  I  S  Q  R  I

 541  CCGAGCCCGCCGAGGGCTGCCCCGCCTGGCTGTGTCAGACTGAAGCCCCATCCAGCCCGT  600
      R  A  R  R  G  L  P  R  L  A  V  S  D  *

 601  TCCGCAGGGACTAGAGGCTTTCGGCTTTTTGGGACAGCAACTACCTTGCTTTTGGAAAAT  660

 661  ACATTTTTAATAGTAAAGTGGCTCTATATTTTCTCTACGCCAAAAAAAAAAAAAAAA
```

Fig. 1. DNA sequence and open reading frame of the HEM45. A full-length open reading frame for HEM45 was determined from cDNA fragments isolated by anchored PCR techniques and from the cloned HEM45 DDPCR fragment. Sequencing was by the dideoxy procedure. Data were compiled and analyzed using the GCG package [15]. The first AUG was assigned to be the initiating methionine residue. The cDNA sequences that were priming sites for the initial differential display product are underlined. The nucleotide sequence of HEM45 is available as GenBank entry U88964.

results for reference probes (36B4 and 1A) using Tina v.2 software (Raytest, Straubenhardt) to quantify radioactivity in gel bands.

Cell culture

Cells were grown in DMEM media (no phenol red) supplemented with non-essential amino-acids, penicillin, streptomycin and 5% Defined Calf Serum (Hyclone, Logan, UT) (DC5) or 5% charcoal stripped serum (sDC5) [18]. pUR transfected cells were generally maintained in puromycin (1 μg/ml), except during the periods of experimental treatment. Cells were grown at 37° in a 5% carbon dioxide atmosphere. E_2 was added to a final concentration of 10 nM with ethanol as vehicle (final ethanol level 0.1%).

Cultures for evaluation of the effects of cycloheximide were removed from puromycin selection 24 h before use. Media were again changed 45 min before addition of E_2, including 0, 10 or 50 μM cycloheximide (Boehringer, Indianapolis, IN).

Animal studies

Animal studies were approved by the Wadsworth Center IACUC and procedures for euthanasia complied with the American Veterinary Medical Association guideline. Wistar rats were 18 to 21 days old and weighed approximately 33 g when used. E_2 was administered by the intra-peritoneal route in 0.2 ml saline (ethanol 0.1%) and the rats were subsequently euthanized by cervical dislocation. Excised uteri were snap-frozen in liquid nitrogen and RNA prepared as described [13]. In a first experiment, E_2 was given at 10 μg/kg body weight in time course experiments and animals killed at time points out to 30 h, using 3 or 4 rats as a single group at each time point. In a second experiment E_2 was given at 0.05 to 1 μg/kg and rats euthanized at 7 h to determine responsiveness to hormone, the matching controls received only vehicle. Three groups of rats, each with three members were used with each treatment.

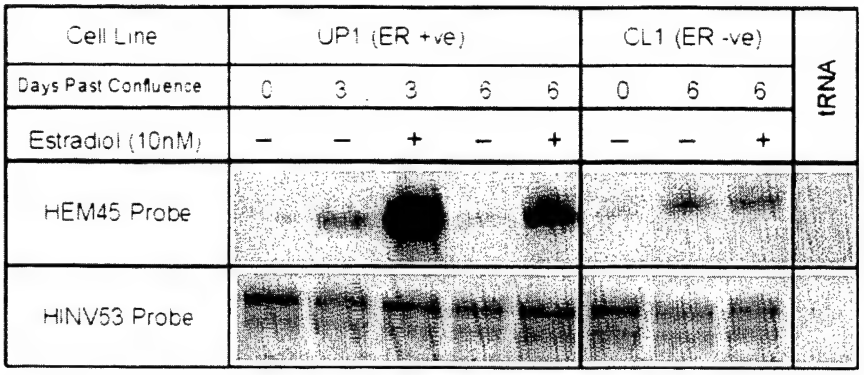

Fig. 2. Detection, by RNase protection assay, of receptor mediated estrogen action on human HEM45 mRNA expression in cultured cells. Untreated UP1 and CL1 cells were harvested at confluence (zero-time controls) and RNA was prepared. Additional groups of cultures were maintained for 3 or 6 days with or without 10 nM E_2 as indicated. RPA's for HEM45 were performed as described in Methods, using an 82-nt fragment of the HEM45 cDNA. HINV53 is a reference control probe derived from an invariant differential display band. tRNA controls were included to determine assay specificity and background.

Isolation of cDNAs to full-length human HEM45

An extended cDNA sequence to HEM45 was prepared by an anchored PCR technique (Clontech, Palo Alto, CA) utilizing double stranded ligated adaptors that provide priming sites only after an initial round of synthesis from an internal gene specific primer [19]. A combination of *Taq* (Perkin Elmer, Norwalk, CT) and *Pfu* (Stratagene, La Jolla, CA) thermostable DNA polymerases was used in PCR reactions to maximize fidelity [20]. Template for the PCR reaction was provided by reverse transcription of total RNA with MMLV-reverse transcriptase from lock-docking dT primers with two degenerate bases at their 3′ ends. Second-strand synthesis utilized *E. coli* DNA polymerase, ribonuclease H and *E. coli* DNA ligase. The RNA was from UP1 cells treated with cycloheximide (50 μM) plus E_2 (12 h, 10 nM). The combined treatment was designed to maximize the levels of expressed HEM45 mRNA (see Fig. 2). Full-length 5′ cDNA's to HEM45 were prepared using sequential nested PCR reactions with anti-sense HEM45 primers representing regions 679–701 and 518–542 of Fig. 3, together with Clontech Adaptor Primers AP1 and AP2, respectively. The product of the second PCR was ligated to T-tailed pCRII. The gene-specific primers were to regions of the original differential display product.

Rat cDNA isolation

A 143-bp fragment (bases 86-229) of TIGR EST 109939 (GenBank H33693) was recovered by reverse transcription-PCR using primers designed from the sequence, and was cloned to pBSK. Poly-dT primed rat lung ds-cDNAs, prepared as described for HeLa cDNAs (see above), were used as PCR template as Northern analysis (not shown) of human tissue RNAs indicated that lung was a relatively rich source of HEM45 mRNA. Antisense RPA probes to rat HEM45 were made with T3 RNA polymerase on Bam HI-linearized template.

DNA sequence analysis

DNA sequence analysis was carried out by conventional dideoxy methodology [21] with denatured plasmid templates and utilizing primers to both common vector sequences and to internal sites. Sequence alignment and analysis utilized programs of the GCG

```
                    1                                          30
HEM45                MAGSREVVA MDCEMVGLGP H.RESGLARC SLVNVHGAVL
RAT EST                        M--T-- QG-V-X---- -V-SYS-D--
XPMC2     TVDKILVKER AFE-LTRT-- -------V-M DGE--I---V -I--LF-KCV
FRT1                 MAPAPER--- ISSVS--V-- RGETDM---V AVIDFT----
CHPGOR    GFVETFKKEL SRDAYPGIY- L----.CYTT -GL-..-T-V TV-DADMR-V

                   50
HEM45     YDKFIRPEGE ITDYRTRVSG VTPQHM..VG ATPFAVARLE ILQLLKGK.L
RAT EST   ---Y----MP -V-----W-- I-R----..HK -I--Q--QK- --K-----.V
XPMC2     ---YVK-TER V-----A--- IR-EDV..KK GE--K-VQK- VSEI-R-R.T
FRT1      L-VYVA-TNP VR---EAKT- IK-EYLYSSR AQDIRAVYQT VR-V-RN-.V
CHPGOR    --T-VK-DN- -V--N--F-- --EADV.AKT SITLPQVQAI L-SFFSAQTI

                        100
HEM45     VVGHDLKHDF QALKEDMSGY TIYDTSTDRL LWREAKLDHC RRVSLRVLSE
RAT EST   ----A-HN-X QX--VR
XPMC2     L---AVHN-L KI-FL-HPKK A-R--QKYKP FKQKVK...S G-P--KL-C-
FRT1      ----SMWL-F MV-GLTHPTK DTR-VALYLP FRNTLRCQ.. -MIG-WT-NY
CHPGOR    LI--S-ES-L L---LIH-.. -VL--AVLFP HYLGFPY... .KR---N-AA

                        150
HEM45     RLLHKSIQNS LLGHSSVEDA RATMELYQIS QR.....IRA R.R..GLPRL
XPMC2     KI-NVKV-TG ..E-C--Q-- Q-A-R--TME KKSWEVA-K- KYT..-VMPV
FRT1      ---GLRCSAA PVD..PL-S- -VALN--RCY AAQWEDT-SS -SWPCE--PP
CHPGOR    DY-GQI--D- QD--N-S--- N-CLQ-VMWK V-QRAQ.-QP -H-SASPAA-

             181
HEM45     AVSD*
XPMC2     DRKSKGPQKD KQCPQ*
FRT1      CFRGCFM*
CHPGOR    -CPWPQAPST TAISPE
```

Fig. 3. Alignment of the human HEM45 open reading frame with related sequences. A multiple sequence alignment of the putative full-length HEM45 ORF with proposed amino-acid sequences for XPMC2, CHPGOR, the rat HEM45 fragment (TIGR 109939 EST) and FRT1 was assembled from GenBank entries using the GCG program 'pileup'. The presented regions of XPMC2 and CHPGOR begin at amino acid residues 217 and 225, respectively. Residues identical to residues in HEM45 are indicated by dashes '-', gaps by periods '.'. Several residues are ambiguous (X) in the rat HEM45 ORF due to unassigned bases in the nucleotide sequence of the EST.

sequencing package [15] with local databases and resources of the National Center for Biotechnology Information.

RESULTS

A differential screen for estrogen regulated mRNAs

Estrogen regulated sequences were identified by DDPCR using RNA from confluent untreated UP1 and control CL1 cells and from cultures of both lines exposed to 10 nM E_2 for five days after confluence. The lines are, respectively, ER-positive (pHE0 transfected) and ER negative puromycin resistant derivatives of the HeLa Cervical adenocarcinoma line (see Methods). An advantage of having cell lines into which ER has been introduced is the availability of matched control cell lines that are identical in all respects except for transfection with ER-α expression constructs.

A 250-bp DNA fragment of an mRNA that is up-regulated by E_2 in the UP1 line, but not in CL1 cells, was identified by DDPCR using the dT primer 5′ T_{12}[C&G&A]G 3′ together with the arbitrary primer OPP13, as described in Methods. This fragment was recovered, cloned and sequenced (bases 440–710 in Fig. 1) as DDPCR product HEM45 (HeLa Estrogen Modulated, band 45). Analysis of full length cDNAs indicates that the OPP13 primer contains mismatches to the HEM45 mRNA sequence at positions one and five (OPP13: 5′ ***G***GAG***T***GCCTC).

An RNAse protection assay for estrogen regulated HEM45

RPAs [22] were used to study the regulation of HEM45 RNAs in ER-positive UP1 and ER-negative CL1 cells. Analysis utilized an antisense probe containing an 82-nt HEM45 fragment (see Methods). Protected bands were detected (Fig. 2) in all UP1 and CL1 samples. Basal levels of HEM45-related bands were detected in RNA from vehicle-treated UP1 and CL1 cells. Levels of HEM45 mRNA were increased in RNA samples from UP1 cells exposed to E_2 for 3 or 6 days. The HEM45 mRNA signal was not elevated in RNA from ER-negative CL1 cells exposed to E_2 for 6 days after confluence, indicating that induction of HEM45 mRNA by E_2 in UP1 cells was mediated by the stably-transfected ER.

The RPA results empirically confirmed the assigned orientation of HEM45 sequences that was originally based on the location of the dT primer in the sequence of the HEM45 DDPCR product (Fig. 1).

Identification of an HEM45 open reading frame

cDNAs encompassing over 700-bp of HEM45 were isolated by anchored PCR methods [19], cloned and sequenced (see Methods).

A 181 amino-acid (aa) residue (M_r 20 300) open reading frame (ORF) was assigned (Fig. 1) commencing with the first AUG triplet of the nucleotide sequence. The region around this AUG has a typical 3-out-of-6 match to the optimal motif of Kozak [23], including a highly favored A at −3. The second and third potential start codons lie in the same frame at +10 and +14 in the peptide sequence (Fig. 1).

Sequence comparisons suggest a relationship of HEM45 to a limited number of genes from diverse sources (Fig. 3), providing a starting point for determining the role of HEM45 protein.

The HEM45 preliminary ORF is roughly the same size as that of Frt1 (192-aa, *ca.* 900-nt mRNA GenBank U21715). The HEM45 and Frt1 ORFs align with the C-terminal halves of longer *Xenopus* XPMC2 (422-aa, xlu10185) and *Gorilla* CHPGOR (526-aa, CHPGOR) entries. Northern blot analysis (data not shown) indicated that the HEM45 mRNA is less than 800-nt, confirming the data from cDNA cloning and excluding the possibility of a longer HEM45 ORF.

Homology to several expressed sequence tags (ESTs) was also noted, but only the rat HEM45 EST homolog is included in Fig. 3 as EST sequence quality is generally too poor for unequivocal assignment of continuous ORFs. Limited homology to several yeast and *C. elegans* sequences was also noted, but these are excluded from Fig. 3 due to lack of known functions; they are accessible through the NCBI Entrez.

Characterization of the HEM45 mRNA estrogen response

A significant increase in HEM45 mRNA levels was seen (Fig. 4) in UP1 cells after three hours of exposure to 10 nM E_2, the earliest time point examined. The HEM45 mRNA level at 3 h was approximately half of the maximal response that was seen at 6 to 12 h. Signal with the HEM45 probe declined by 24 h to one-third of maximal values, but was still elevated relative to the zero hour control.

The maximal estrogen induction of HEM45 mRNA levels at 6 to 12 h (Fig. 4), rather than at earlier times, left it unclear whether the response was a direct or a secondary response to hormone treatment. Cultures were therefore treated with the protein synthesis inhibitor cycloheximide [24] to prevent secondary estrogen responses in the UP1 cells. Treatment of UP1 cultures with cycloheximide (10 or 50 μM) for three or twelve hours increased basal levels of HEM45 mRNA as compared to levels in vehicle-treated cultures that did not receive cycloheximide (Fig. 5). HEM45 mRNA levels were superinduced by E_2 following cycloheximide pretreatment (Fig. 5). The failure of cycloheximide treatment in blocking E_2-induction of HEM45 mRNA levels indicates that HEM45 transcription is directly regulated by estrogens via nuclear ER-α.

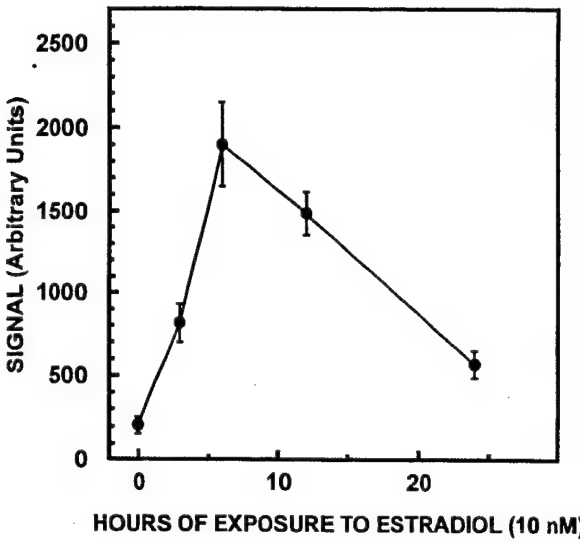

Fig. 4. The temporal response of human HEM45 mRNA expression to estradiol treatment. UP1 cells in T25 flasks were treated with 10 nM E_2 or vehicle (0.1% ethanol) in fresh DC5 media as they approached confluence and were harvested at the indicated time points. Points represent the means ± SE for RPAs on samples from three flasks. Data were collected with a Fuji-Bas Imager and the signal for HEM45 was normalized to that of the 36B4 reference probe.

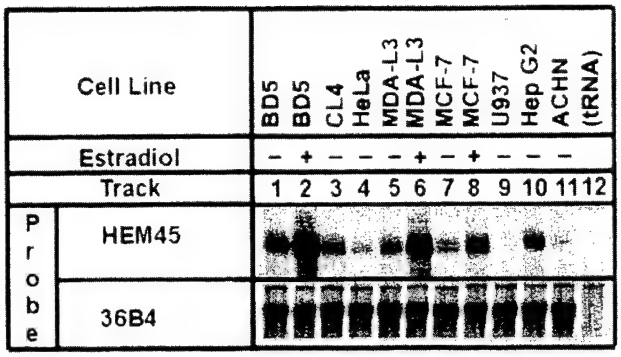

Fig. 6. HEM45 mRNA expression in selected human cell lines. HEM45 expression was evaluated in a panel of additional cell lines. E_2 action was determined in cell lines known to carry functional ER by treatment of parallel cultures with vehicle or 10 nM E_2 for the 48 h prior to harvesting. BD5: An additional ER-positive HeLa line, CL4: An ER-negative HeLa derivative. MDA-L3: ER-positive derivative of ER-negative MDA MB-231 breast cancer cell line. BD5, CL4 and MDA-L3 are stably transfected lines selected for puromycin resistance (see Methods). HeLa: Parental HeLa line. MCF-7: naturally ER-positive breast cancer line. U937: Monocyte-like tumor line. HepG2: Liver derived tumor line. ACHN: Kidney derived tumor line. RPAs utilized an 82-bp HEM45 antisense transcript fragment and a 200-nt 36B4 reference probe. tRNA controls were included to demonstrate assay specificity.

Expression of HEM45 in a battery of cell lines

Protected fragments were detected (Fig. 6) with HEM45 probes in RPAs carried out with RNA from the parental HeLa line, an additional puromycin-resistant HeLa-derived control line, CL4, and an additional ER-positive HeLa-derived line, BD5. Detection of the mRNA in the parental HeLa line (Fig. 6) indicates that HEM45 is normally expressed in HeLa and is not derived from genes encoded by the transfected ER and puromycin expression vectors. HEM45 mRNA levels were elevated in RNA from estrogen-treated (10 nM, 48 h) ER-positive BD5 cells (Fig. 6), as seen previously for the ER-positive UP1 HeLa-derived line (see Figs 2 and 5). The similarity of responses in BD5 and UP1 cells confirms the generality of the responses in HeLa on conversion to an ER-positive phenotype.

HEM45 was detected in all of four additional adherent permanent cell lines, including two breast-cancer-derived lines (ER-positive MCF-7 and ER-positive MDA-L3 (pHE0 transfected MDA-MB-231, see Methods), together with liver- and kidney-derived lines (HepG2 and ACHN, respectively). The HEM45 signal was extremely weak, but detectable, with U937 monocyte-related cells which are grown in suspension. HEM45 mRNA levels were increased by E_2 treatment (10 nM, 48 h) of the ER-positive breast-cancer line MCF-7 and the ER-positive MDA-L3 derivative of MDA-MB-231. This suggests that HEM45 mRNA provides a ubiquitous marker for ER-mediated estrogen action.

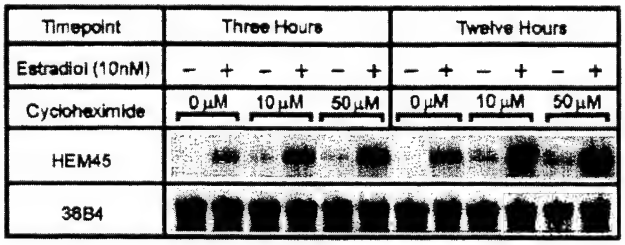

Fig. 5. Expression of human HEM45 mRNA in UP1 cells under conditions of protein synthesis blockade by cycloheximide. UP1 cells were placed in puromycin free DC5 media as they neared confluence. Media was changed again after 12 h and cycloheximide at 0, 10, or 50 μM was added. Forty-five minutes later cells received 10 nM E_2 or ethanol vehicle (0.1%), and were harvested at 3 or 12 h after addition of steroid. RNA was prepared as described in Methods, and samples analyzed for HEM45 and 36B4 mRNAs by RPA.

Rat HEM45 mRNA expression in the immature uterus

A rat model for sequences related to HEM45 was established to study estrogen regulation *in vivo*. A survey of the GenBank EST directory identified an otherwise uncharacterized rat sequence with high homology to human HEM45 that had been acquired in a survey of cDNAs from NGF-treated PC12 cells. The 270-bp rat EST 109939 has 68% identity to human HEM45 at the nucleic acid level by FastA analysis, and 65.5% identity over 87 amino acid residues (Rat HEM45 ORF, Fig. 3), higher values than seen between human HEM45 and other sequences. A 143-bp RT-PCR fragment to the rat EST was recovered as described in Methods, and is referred to as

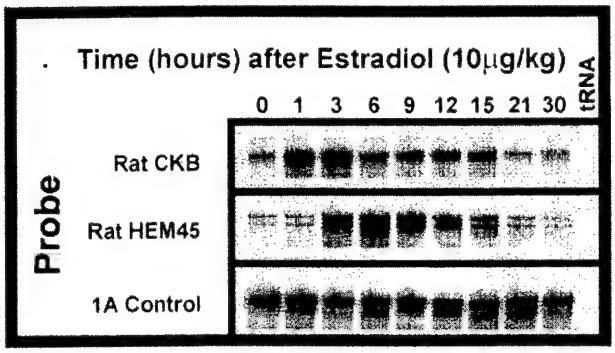

Fig. 7. Temporal expression of rat uterine HEM45 following estrogen treatment. Wistar rats, 18–21 days, ~33 g, were used in groups of 3 to 4. E_2 was administered IP at 10 μg/kg in 0.2 ml saline (ethanol 0.1%), and animals were euthanized at the indicated times. The control group was untreated. RNA was prepared as described [13] and 10 μg samples analyzed using rat HEM45, rat CKB 3′, and rat 1A (reference) probes in RPAs.

'rat HEM45'. The fragment has sequence identical to the 86-229 region of EST 109939.

Temporal regulation of rat HEM45 mRNA expression was evaluated following E_2 administration at 10 μg/kg body weight which elicits a maximal response of CKB mRNA in the one- to three-hour time period [13]. The temporal pattern of rat HEM45 mRNA expression in the estrogenized immature uterus was distinct from that for CKB steady state mRNA levels (Fig. 7) and from that reported for 'early-response' genes such as c-jun [25], c-fos [26], and zif-268 [27]. Rat HEM45 mRNA levels were unaltered at one hour after E_2 treatment (Fig. 7). Levels were increased at three hours and peaked in the six- to nine-hour period, returning to basal levels by 21 h. Maximal induction of greater than 8-fold, determined by quantification of Phosphorimager data, was seen at six hours after estrogen administration. The temporal pattern of rat HEM45 mRNA expression in the estrogenized uterus is very similar to that seen for the human sequence in UP1 cells (Fig. 4). The estrogen responsiveness of rat HEM45 mRNA expression was in contrast to the invariant expression of the control sequence 1A.

Sensitivity to E_2 was also determined for rat HEM45 in the uterus, using a single 7 h time point where HEM45 mRNA induction should be close to maximal. Induction of rat HEM45 mRNA levels was seen at low levels of E_2, with a half-maximal response at *ca.* 0.05 μg/kg body weight (Fig. 8). HEM45 mRNA induction plateaued by 0.3 μg/kg body weight.

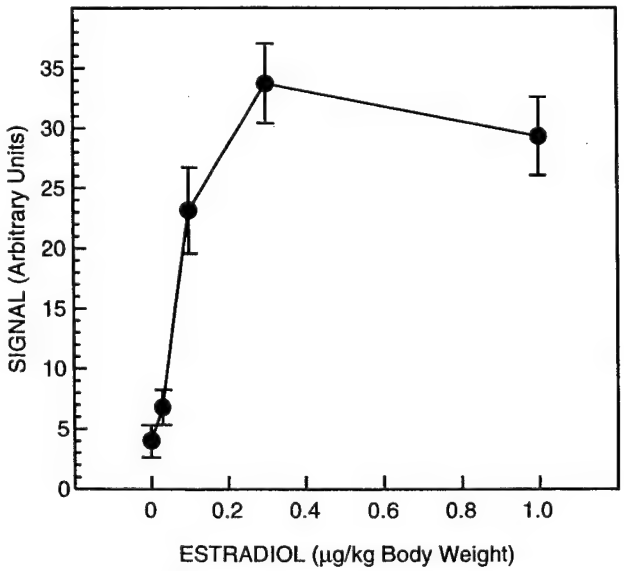

Fig. 8. Rat uterine HEM45 mRNA expression under conditions of varying estradiol concentration. Rats were sacrificed at 7 h after treatment. The indicated amounts of E_2 in saline (ethanol 0.1%) were administered as described in the legend to Fig. 7; controls received vehicle alone. RNA was isolated and analyzed as described in Fig. 7. RPA data for the rat HEM45 and rat 1A reference mRNAs were collected and quantified using the Fuji-BAS Imager. Results for HEM45 were normalized to results for the 1A reference probe.

DISCUSSION

This study describes the characterization of HEM45, a novel estrogen regulated gene identified by DDPCR. It demonstrates the utility of a reconstructed, E_2-regulated system employing cells stably transfected with ER-α in the study of estrogen action. The ER-transfected HeLa derivatives provide an ER-positive cell model for the human reproductive tract, for which there are few effective naturally ER-positive models. The availability of matched ER-negative lines provides a powerful control allowing total elimination of the basal influence of residual serum estrogens which could affect interpretation of results from the highly sensitive differential display assay. The use of reconstituted ER-positive cell models should also lead to clarification of the roles of the alternative ER genes [2].

Recent rapid expansion of the sequence databases due to development of sequence-tag strategies has aided rapid identification of the rat equivalent of human HEM45. This allowed confirmation of the *in vivo* expression and estrogen regulation of the gene in the uterus.

Levels of the HEM45 mRNA are modulated by estrogen in UP1 ER-positive, HeLa-derived cells subjected to either short (Fig. 4) or long-term exposure (Fig. 2) to E_2. Twenty-fold increases in HEM45 mRNA levels were detected under optimal conditions. E_2 action is mediated by the nuclear ER, as levels were not elevated by E_2 in matching ER-negative CL1 control cells. Eight-fold induction of HEM45 by E_2 was seen in the rat uterus, but it remains to be determined in which layers this response is occurring. The insensitivity to cyclohexi-

mide of the stimulation of HEM45 mRNA levels by E_2 in UP1 cells and the high sensitivity to E_2 in the uterine model indicate that HEM45 induction is a primary estrogen-modulated event, despite the delay in response in comparison with some 'immediate-early' genes.

The half maximal induction of HEM45 is estimated to occur at 0.05 μg/kg E_2. This is a much lower dose than is required for half maximal stimulation of uterine CKB mRNA (5–7 μg/kg) [13] and lower than the amounts required for induction of 'early-response' genes. Insignificant induction of c-fos [26] and zif-268 [27] expression was reported with a low dose of 0.04 μg/kg E_2. The c-fos, c-jun, and zif-268 mRNAs encode components of transcription factors which are estrogen inducible in the uterus of ovariectomized immature rats. These mRNAs are elevated in the 1- to 4-h period following estrogen treatment and have been termed 'early-response' genes [28] and proposed to generate competence factors that mediate the early stage of the uterine response to estrogens. Indeed, it is possible that, in the naturally cycling rat, HEM45 mRNA levels could increase *before* genes such as c-fos and zif-268 as a response to circulatory estrogens, at a lower hormone level and hence earlier point. This suggests that HEM45 induction is not a secondary response to the induction of 'early-response' gene induction, a supposition directly supported by the failure (Fig. 5) of protein synthesis inhibitor cycloheximide to block HEM45 mRNA induction in UP1 cells.

HEM45 is a novel gene; however, the action of the related *Xenopus* XPMC2 sequence in *S. pombe* [29] and the capacity of Bracket Fungus Frt1 gene [30] to cause fruiting-body development suggest that HEM45 may have a role in mediating regulatory or developmental aspects of estrogen action.

The *Xenopus* ovary XPMC2 cDNA sequence [29] can rescue mutant *S. pombe* from a mitotic catastrophe phenotype that was created by disruption of cdc2 kinase regulation by eliminating Wee 1 and Mik 1 kinase activities [31]. Control of cdc2 kinase is a key step in controlling cell-cycle transition from G1 to S phase and G2 to M [32], but the point of XPMC2 action is unknown. XPMC2 does not cause the inhibitory tyrosine phosphorylation on cdc2 kinase [29], suggesting that it is not simply replacing Wee 1 and Mik 1 kinases in the rescued *S. Pombe* mutant. Frt1 from the basidiomycete *Schizophyllum commune* (Bracket Fungus) causes fruiting-body formation when introduced to suitable homokaryons, bypassing the need for fusion of two haploid strains of opposite mating types [30].

The N-terminal 9 to 30 region of the HEM45 ORF is relatively well conserved in the multiple sequence alignment, particularly with respect to XPMC2, suggesting a conserved structure/function for this region. Several mutations in the N-terminal region of *Frt1* abolished its capacity to cause fruiting-body development [30] and a potential dinucleotide binding motif is noted for the N-terminal region of *Frt1*.

HEM45 and Frt1 have lower similarities in FastA comparisons than HEM45 has to the *Xenopus* and *Gorilla* sequences (Fig. 3) despite similarities in length of HEM45 and Frt1 ORFs. FRT1 and the HEM45 ORF showed 29% identity in a 166-aa overlap as compared with 39% identity of XPMC2 and the HEM45 ORF in a 163-aa overlap. HEM45 is not, however, the mammalian analogue of XPMC2, as a human EST (R02224) has greater homology to the *Xenopus* sequence with 60% identity in a 106-aa overlap.

The *Xenopus* XPMC2 and *Gorilla* CHPGOR proteins are nuclear [29, 33] but the putative nuclear localization signals are outside of the regions aligning with Frt1 and the HEM45 ORF, making their cellular location problematic on the basis of sequence comparisons.

In-vivo studies focused on estrogen regulation in rodent female reproductive tract, but HEM45 mRNA was ubiquitously expressed in human cell lines and estrogen regulation of HEM45 mRNA levels was also found in breast-cancer cells. The role of estrogens in control of HEM45 in breast and 'non-target' tissues needs to be developed but expression and regulation by estrogen in both rodent and primate systems suggests a conserved function for HEM45-related sequences. It also remains to be determined whether regulation of HEM45 is unique to estrogens or is relevant to the action of other steroid and peptide hormones in reproductive physiology.

Acknowledgements—ER-positive HeLa cell lines were isolated with the careful help of Hillary E. Sussman. The work presented here was greatly aided by the staff and resources of the Wadsworth Center Molecular Genetics, Molecular Immunology, and the Computational Biology core facilities. Rats were bred and maintained by the staff of the Griffin animal production unit. Figures were prepared by the Visual Media Unit. Support was provided by NIH grant DK-41706 and army contract DAMD17-96-1-6183.

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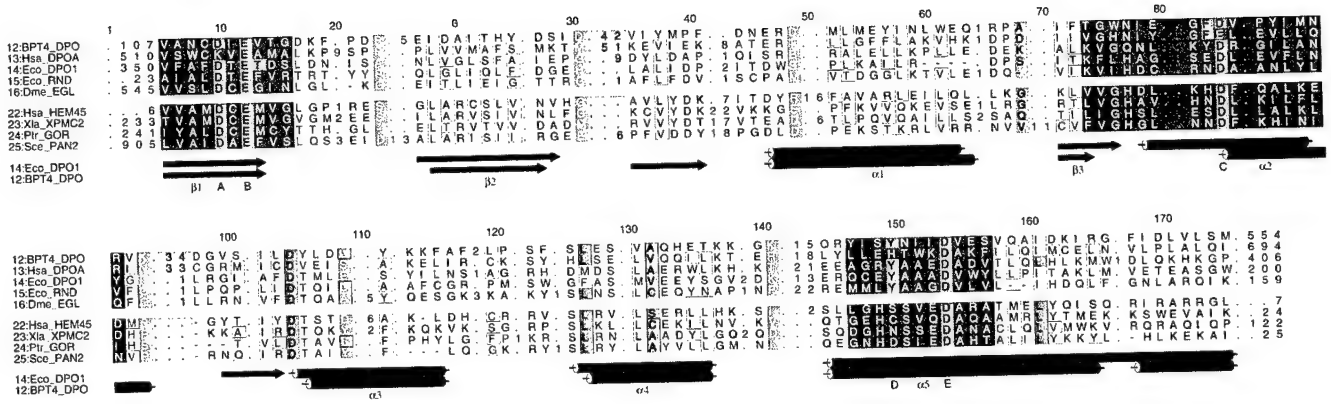

Figure 1. HEM45 has sequence motifs relating it to the proofreading 3' 5' exonuclease of E.coli DNA Pol I

Data excerpted from color Fig.1 of Moser et al (Nucleic Acid Res 24:5110-18,1997). The three largest shaded blocks (residues 4-15, 71-90, 147-158) represent the conserved 'exo domains' recognized in Polymerase exonucleases. Lighter shaded areas mark major gaps for some seqs. Within the exo motifs are strongly conserved acidic (Asp/Glu) residues, tagged ABCE under the alignment. These coordinate with metal ions in the crystalized Pol1 molecule. The active site in the Pol 1 exonuclease is the tyrosine (Y) at 150 (tagged D under seq). This is missing in the HEM45 subfamily; probably replaced by the histidine (H) at the preceding position.

12. Bacteriophage T4 pol proof-reading subunit. 13. Human DNA pol α proof reading domain, 14. E.coli Pol1 14 E.coli RnaseD15. Secondary structures of 12 and 14 are shown below the alignment. These are derived from solved structures.

Sequences 22-25 form a subgroup. This is the HEM45 family that we previously recognized. The yeast Rnase is an addition, and suggests this group may have nuclease activity.

22. HEM45. 23. XPMC2: Xenopus seq, rescues cell cycle control defect in S. pombe model. 24. CHPGOR gorilla nuclear auto-antigen. 25. PAN2 *S cerevisiae* polyA shortening RNase subunit.

Subcellular localization of HEM45 is regulated during the cell cycle

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ABSTRACT

The HEM45 gene encodes a protein with homology to the Xenopus XPMC2 gene which complements yeast Wee1 kinase and Mik1 kinase genes. These yeast genes modulate cyclin associated Cdc2 kinase activity required for normal yeast mitotic division. The homology that HEM45 shares with XPMC2 may imply that HEM45 is associated with cell cycle regulation. Therefore, we examined the cellular level and subcellular location of HEM45 in HeLa cells. Antibodies against HEM45 peptide sequence 3-16 and against bacterially expressed recombinant protein recognized a polypeptide of 20,300 Mr in HeLa cell lysates. Indirect immunofluorescence microscopy showed that the subcellular location and level of expression of HEM45 changed dramatically as cells progressed from interphase to mitosis. During interphase, HEM45 was found exclusively in the cytoplasm. As cells entered mitosis, HEM45 accumulated at the cytoplasmic side of the nuclear envelope. With breakdown of the nuclear envelope, HEM45 was redirected to the mitotic spindle in metaphase and anaphase. In telophase HEM45 was found randomly distributed throughout the cell body. HEM45 was not found associated with microtubules of interphase cells. By Western blot analysis, mitotic cells expressed approximately three-fold more HEM45 protein than interphase cells. Changes in expression and distribution of HEM45 i.e., its association with the mitotic apparatus, suggest that HEM45 complements cell cycle regulatory proteins, or influences mitotic spindle fiber dynamics.

INTRODUCTION

The cDNA of HEM45 mRNA was characterized independently by Pentecost (1998) and Gongora et al. (1997), who referred to it as IGS20 mRNA. HEM45 transcription is regulated by both estrogen (Pentecost,1998) and interferon (Gongora et al., 1997). The open reading frame (ORF) of HEM45 cDNA encodes a 181 amino acid polypeptide with a predicted M_r of approx. 20.3 kDa (Pentecost, 1998; Gorgora et al., 1997). A search for amino acid sequence homologies in the EMBL SwissProt database using the program FASTA found 54% homology between HEM45 peptide sequence 1-60 and the *Xenopus laevis* XPMC2 gene product (Su and Maller,1995; Pentecost,1998). Expression of the XPMC2 gene product rescues fission yeast *Schizosaccharomyces pombe* Wee1 and Mik1 kinase mutants displaying a mitotic catastrophe phenotype. Because of its sequence homology to XPMC2, it is plausible that HEM45 may influence cell cycle regulation (Su and Maller,1995).

In mammalian cells, the cell cycle is controlled by a network of regulatory enzymes that coordinate the timing and execution of each stage of the cell cycle (for reviews see Murray, 1992 and Hartwell and Weinert, 1989). For the most part this regulatory network is controlled by a group of related enzymes known as the cyclin dependent kinases (cdk) (for review see Nigg,1993). The activity of these enzymes is controlled in part by their association with regulatory subunits called cyclins (Nigg, 1993). A number of cyclin cdk complexes comprise the network that regulate cell cycle progression. The activity of cyclin kinases is dependent on post-translational modifications, which include a series of phosphorylation and dephosphorylation events (Simanis and Nurse,1986; Booher and Beach,1986, Hagan et al.,1988; Ducommun et al.,1991; Gould et al.,1991).

In yeast, entry into mitosis requires the activity of an M-phase-promoting factor (MPF), a complex of a cyclin dependent kinase cdc2, and a B-type cyclin (Murray,1989; Pines and Hunter,1990; Rong and Murray,1991). Activity of the cyclin B cdc2 complex is controlled by phosphorylation/

dephosphorylation of cdc2 and cyclin B levels. MPF is inactive when Thr14 and Tyr15 of cdc2 are phosphorylated by Wee1 and Mik1 kinases (Gould and Nurse,1989; Lundgren et al.,1991). At the end of G_2, these cdc2 sites are dephosphorylated by phosphatase cdc25 and the cdc2 cyclin B complex is active.

Fission yeast *Schizosaccharomyces pombe* lacking both Wee1 and Mik1 kinase activities enters mitosis before completion of DNA synthesis and displays a mitotic catastrophe phenotype. The Xenopus XPMC2 gene product, to which HEM45 displays its best homology (Pentecost, 1998; Gongora et al., 1997), can rescue Wee1, Mik1 yeast mutants (Su and Maller,1995). How the XPMC2 gene product prevents the catastrophe phenotype is not clearly understood; however, it may act as a negative cell cycle regulator by acting as a substrate for cdc2 kinase phosphorylation (Su and Maller, 1995).

Typically, the level of expression of cell regulatory proteins changes dramatically during the cell cycle (for review see Pines,1995), and several examples may be cited where subcellular compartmentation is associated with regulation of cyclin cdk complexes (Riabowol et al., 1989; Pines and Hunter, 1991; Baldin and Ducommun, 1995; Girad et al., 1992).

Cdc2 accumulates in the nucleus (Riabowol et al.,1989), while the cyclin B subunit, to which it complexes, accumulates in the cytoplasm in G_2. At the beginning of mitosis, cyclin B translocates into the nucleus where it complexes with cdc2, becomes active (Pines and Hunter,1991), and interacts with the nuclear lamin and spindle apparatus. The activity of Wee 1 kinase, which negatively modulates cdc2 kinase, changes as it relocates to the mitotic equator and to the midbody in mitosis (Baldin and Ducommun,1995). The Wee1 counterpart, cdc25 phosphatase, is a nuclear protein during interphase, but activates cyclin cdc2 complexes on spindle fibers after the nuclear envelope dissolves (Girard et al.,1992).

We postulated that HEM45 may be involved as a regulatory protein of the HeLa cell cycle. To test this hypothesis, we investigated the levels and cellular distribution of HEM45 in HeLa cells during the cell cycle. In this report, we show that the levels and subcellular location of HEM45 change in HeLa cells during the cell cycle. Its subcellular distribution, and its apparent association with spindle microtubules, reveal likely biological functions for HEM45.

MATERIALS & METHODS

Expression of Recombinant HEM45 protein

cDNA to HEM45 mRNA (Pentecost,1998) was made using reverse transcriptase with poly-dT and the complete HEM45 ORF amplified by polymerase chain reaction (PCR) with primers that permitted in-frame insertion into the expression vector pET-15b (Novagen, Milwaukee,WI). The pET 15b vector allows expression of inserted sequences under control of an IPTG-inducible T7 promotor. The expression cassette encodes a Met initiator codon followed by a thrombin cleavable leader sequence containing a poly-Histidine sequence for metal chelation chromatography of expressed protein.

Recombinant (rec) HEM45 was expressed in *E.coli* transformed with the expression vector pET-15b (Novagen, Milwaukee,WI). Four hours after induction with 2 mM IPTG, cells were harvested. Inclusion bodies containing insoluble HEM45 protein were enriched by resuspending cells in cold 5 mM imidazole, 500 mM NaCl, 20 mM Tris pH 7.9 buffer, and sonicating the suspension three times to release soluble protein. Inclusion bodies were harvested by centrifugation at 20,000 xg for 15 min, collecting a pellet that was washed two additional times as described. After the final wash, inclusion bodies were solubilized by resuspending the pellet in cold 8M urea in the same buffer. The resuspended inclusion bodies were sonicated and kept at 4°C. The suspension was clarified by centrifugation at 25,000 rpm for 20 min in a Ti Sw55 rotor. The supernatant containing amino

terminal His_6-tagged rec HEM45 protein was passed over a Ni^{2+} charged His-bind resin (Novagen,Milwaukee, WI). The affinity column was washed with 8 M urea, 20 mM imidazole, 500 mM NaCl, 20 mM Tris pH 7.9, and the HEM45 eluted with 8 M urea, 300 mM imidazole, 500 mM NaCl, 20 mM Tris pH7.9. To determine the molecular weight of the rec HEM45 without its histidine leader, the protein was digested with 1.5 units of thrombin for 30 min at 37° C, and then subjected to immunoblot analysis.

Production and Characterization of HEM45 Antibodies

Gray Flemish giant chinchilla rabbits were immunized with gel-purified rec HEM45 protein possessing an amino terminal Histidine leader (Rabbit Y29) or a synthetic peptide (Rabbit X826) corresponding to HEM45 sequence 3-16 (^{3}GSREVVAMDCEMVG^{16}) as predicted from HEM45 cDNA (Pentecost, 1998). For the purpose of preparing antiserum to HEM45, column-eluted HEM45 was further purified on a preparative SDS-15% polyacrylamide gel, the band was sliced from the gel, HEM45 was electroeluted, and used as an immunogen. The synthetic peptide was coupled to ovalbumin through an additional carboxyl terminal cysteine not present in the native sequence. (Weiner and Dias, 1991). Rabbits were immunized four times, bi-weekly, with either one milligram of peptide equivalents or one milligram of rec HEM45 protein. Serum collected after the fourth immunization was cleared by caprylic acid and ammonium sulfate precipitation (Harlow and Lane, 1988).

Cell Culture

HeLa cells were originally obtained from the American Type Culture Collection (Rockville, MD) and maintained in culture by the Wadsworth Center's tissue culture facility. Cells were grown in modified Eagle's medium (MEM), pH 7.2, containing 10% fetal bovine serum, and maintained at 37°C in 5% CO_2 atmosphere.

For microtubule disassembly, the cells were either pre-incubated for one hour in medium at 4°C, or with 50 μM nocodazole at 37 °C, prior to fixation at 4°C.

Synchronization

HeLa cells were synchronized with a double thymidine block. Exponentially growing cells were arrested in S phase by treatment with 2.5 mM thymidine for 16 h, and were released from the arrest by washing with fresh medium. Cells were grown in fresh medium for 9 h and then retreated with 2.5 mM thymidine for 16 h. More than 40% of the cells had a rounded mitotic phenotype 10 h after release from the double thymidine block. Flasks were gently tapped to release mitotic cells, which were counted and then harvested by centrifugation.

Fluorescence Immunohistochemistry

For immunofluorescence staining, HeLa cells grown were on 15-mm diameter glass cover slips, rinsed at 37° C with Small's cytoskeleton stabilizing (cs) buffer (Small, 1981)[137 mM NaCl, 5 mM KCl, 1.1 mM $Na_2HPO_4.7H_2O$, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, 5.5 mM glucose, 5.0 mM PIPES, 2 mM $MgCl_2.7H_2O$, 2 mM EGTA, pH 7.2], and fixed at 37°C for 5 min with 3.7% formaldehyde (in cs buffer). Fixed cells were permeabilized for 20 min in 0.5% Triton X-100/ cs buffer and then rinsed for 10 min with 10 mg/ml glycine in cs buffer. After the cells were blocked for 1 h with 0.2% normal serum in cs buffer, they were incubated for 1 h at RT with primary antibodies diluted in 0.1% Tween-20 in cs buffer. The antibody dilutions used were 1:500 for X826, 1:100 for Y29, and 1:100 for mouse anti-tubulin monoclonal antibody (Sigma, St. Louis, Mo.). Specificity of staining was assessed as follows: Peptide challenge experiments were conducted with X826 anti HEM45 peptide 3-16 antiserum. Anti peptide antiserum was incubated overnight at 4°C with 10μg of immunizing peptide per ml of serum. Prior to addition to the serum, peptide was dissolved in a minimal volume of water. The control for Y29 anti rec HEM45 protein was preimmune serum of that animal. In the case of

either treated or control cells, cells were washed, then challenged for 1 h at RT with either fluorescein isothiocyanate (FITC) or rhodamine (TRITC)-conjugated secondary antibody (Organon Teknika, Durham, NC). Immunostained cells were mounted in 50% glycerol containing 1 mg/ml -propyl-gallate, viewed, and photographed on a Nikon-epifluorescent microscope.

Immunoblot Analysis.

Nonsynchronized HeLa cells were removed from culture flasks by washing with phosphate buffered saline containing 0.5 mM EDTA. The cells were harvested by centrifugation, counted, and solubilized in electrophoresis sample buffer. Samples were boiled for 5 min before electrophoresis on SDS/15% polyacrylamide gels (Laemmli,1970). PAGE resolved polypeptides were electro-blotted for 1 h at 0.5 amps in Towbin's buffer (Towbin et al., 1979) onto immobilon P membranes (Millipore, Bedford,MA). Protein blots were probed with HEM45 antibodies, and then processed for detection of second antibody by ECL (Amersham, Arlington Heights, IL) or Western blue reagent (Promega, Madison,WI). Specificity of detection of HEM45 for each antiserum was tested as follows:HEM45 Western blots were probed with either Y29 (anti-rec HEM45) immune or pre-immune serum. Western blots with X826 (antipeptide) antiserum were compared to pooled non-immune rabbit serum. Results from ECL Western blot detection were quantitated with an Image Master Scanner/ Densitometer (Pharmacia, Piscataway, NJ).

RESULTS

Characterization of recombinant HEM45 by western immunoblot analysis

HEM45 was expressed in bacteria as a fusion protein of the 181 amino acid HEM45 polypeptide and a 20 amino acid amino-terminal leader including 6 histidine residues. The oligohistidine amino terminal leader allowed rapid partial purification of rec HEM45 using metal chelation chromatography (Fig. 1). The amino terminal leader possessed a thrombin cleavage site, which allowed removal of

seventeen of the twenty amino acids of the leader. As shown in figure 1.A, lane 2, digestion of recombinant HEM45 with thrombin produced a polypeptide of approx. 20 K Da, as predicted by HEM45 cDNA (Pentecost, 1998; Gongora et al., 1997). Both thrombin digested and undigested rec HEM45 protein were immuno-reactive (Fig.1.B) by Western analysis using antiserum X826 against the HEM45 peptide. These data confirmed that we expressed rec HEM45 protein in bacteria and generated an anti-peptide antiseum that recognized HEM45 protein. Because non HEM45 bacterial proteins co-eluted with authentic HEM45, it was necessary to further purify HEM45. SDS-polyacrylamide gel electrophoresis followed by electroelution allowed isolation and use of HEM45 as a separate immunogen. Both anti-peptide and anti-rec HEM45 antisera recognized a polypeptide of approximately 20 kDa in HeLa cell lysates (Fig.2). Figure 2B shows that antibody Y29, made against rec HEM45, detects HEM45 in HeLa cell lysates. Pre-immune Y29 serum produced similar background staining but no specific HEM45 staining (data not shown). Anti-peptide antiserum X826 (Fig.2A) also detected authentic HEM45 in HeLa cell lysates. However, because the peptide was coupled to ovalbumin for immunization, background staining was observed at high molecular weights. This non-specific staining could be abrogated by inclusion of 10 $\mu g\ ml^{-1}$ of ovalbumin with X826 (data not shown). Although both antisera were useful for immunohistochemistry (see Fig. 3), Y29 was used for most of the studies.

Localization of native HEM45 in HeLa cells

Both X826 and Y29 antisera produced nearly identical staining patterns in interphase HeLa cells (Fig.3.A,B). Both antibodies produced a weak, speckled staining pattern localized to the cytoplasm. This staining was specific since no staining was observed with Y29 pre-immune serum (Fig.3.D) or when the X826 was blocked by incubation with the immunizing peptide (Fig.3.C). In this experiment X826 was not preabsorbed with ovalbumin, yet minimal background staining was observed. At low

magnification (Fig.3.E), a dramatic difference in the staining intensity of interphase and mitotic cells (white arrows) was witnessed. In this figure an interphase cell is seen in the upper left corner (see also Figure 3B).

To increase the number of mitotic cells in cell cultures, HeLa cells were blocked in the G_0 /S transition with a thymidine block, released, and fixed 12 hours later. Under these experimental conditions, mitotic cells represented greater than 50% of the cell population, judged by chromosome morphology (Data not shown). In interphase, HEM45 staining was weak and exhibited a fine speckled staining pattern randomly distributed throughout the entire cytoplasm (Fig.4.A). No detectable HEM45 staining was found in the nucleus. When chromosomes began to condense at early prophase (Fig.4.B), HEM45 staining became more punctuate and appeared to concentrate at the cytoplasmic side of the nuclear envelope. This apparent redistribution of HEM45 may have been influenced by cell rounding. At metaphase (Fig.4.C), condensed chromosomes appeared negatively stained, and diffuse amorphous HEM45 staining overlaid the region of the mitotic apparatus. In late anaphase (Fig.4.D), HEM45 could be observed on the spindle caps. HEM45 again became randomly distributed throughout the cytoplasm of daughter cells and the connecting midbody bridge in telophase.

Comparison of HEM45 and tubulin location in HeLa cells

Since the preceding immunological data suggested that HEM45 was associated with microtubules of the mitotic apparatus, HeLa cells were double-immunostained with Y29 and mouse monoclonal antibody to tubulin. To avoid cross-reactivity between antibodies, cells were stained with one primary antibody at a time. Figure 5 shows tubulin-stained spindle fibers of a metaphase cell (Fig.5.E) and the location of HEM45 (Fig.5.D) in the same cell. While the structure stained by HEM45 antibody is poorly defined, it does resemble the general outline of the mitotic spindle apparatus. Cells stained in the reverse order (i.e. tubulin antibody, then HEM45 antibody), produced

the same staining pattern (data not shown).

It was the possible that HEM45 staining of spindle fibers was the result of "image bleed through", resulting from overlap in the excitation wavelengths and the barrier filters used. To address this point, cells stained with a rhodamine secondary antibody were examined for "image bleed through" at the fluorescein wavelength. No signal was detected (data not shown).

Interphase cells displaying extended cytoplasm and well defined individual microtubules were studied to determine if HEM45 is associated with microtubules. Comparison of tubulin staining (Fig.5.B) and HEM45 staining (Fig.5.A) failed to detect HEM45 localization on microtubules. These data suggest that HEM45 association with microtubules occurs in mitosis. Cells were treated with the microtubule-depolymering drug nocodazole (20 μM) to test whether the HEM45 staining found in Figures 4.C and 5.D was microtubule dependent. Nocodazole disruption of spindle fibers produced uniform HEM45 staining throughout the ctyoplasm of mitotic cells (Fig. 6.A), supporting the concept that spindle fibers serve as a framework for HEM45.

We attempted to repeat these results after depolymerizing the microtubules by incubating the cells at 4°C. Cold treatment for short periods of time (less than ten minutes), had little effect on mitotic spindle fibers, but as the duration of cold treatment increased, spindle fibers shortened and decayed. Those most resistant to cold treatment were spindle fiber remnants found in the midbody bridge of telophase cells (Brinkley, 1975). For our studies, cell cultures were incubated on ice for 30 minutes, then fixed at 4°C (Figure 7). In contrast to normal and nocodazole treated, cold treated mitotic cells showed HEM45 staining at the midbody furrow between daughter cells (Fig.7.B). Typically, HEM45 was dispersed randomly throughout the cytoplasm of telophase cells, and was not focused at midbody furrow (see Fig.4.E). Approximately 30-40% of HEM45 staining associated with telophase cells after cold treatment was localized to the midbody furrow.

Characterization of native HEM45 in Proliferating Cell Cultures

Immunostaining for HEM45 is high during mitosis and low in interphase. (See Fig. 3). This could reflect different levels of HEM45 expression or differential accessibility of this protein at different stages of the cell cycle. To explore this aspect further, the level of HEM45 in mitotic and interphase was examined by western blot analysis. Mitotic cells were harvested 10 h after the release of a double thymidine block. Approximately 50% of the harvested cells were mitotic by DAPI staining. Interphase cells were harvested from a nonsynchronous proliferating cell culture. Figure 8 displays an western immunoblot of mitotic and interphase cell lysates. Based upon densitometric analysis of the immunostained HEM45 band, mitotic cells exhibit three-fold more HEM45 than interphase cells.

DISCUSSION

In this series of experiments, we examined the levels and subcellular distribution of HEM45 protein in HeLa cells. HEM45 is: (i) randomly distributed throughout the cytoplasm during interphase; (ii) concentrated at the nuclear envelope at prophase; (iii) colocalized with elements of the mitotic apparatus in metaphase and anaphase, (iv) redistributed throughout the cytoplasm in telophase, and (v) expressed at higher levels in mitotic cells than in interphase cells.

Immunofluorescence microscopy (Fig.3.E) revealed that mitotic cells stained more intensely for HEM45 protein than interphase cells. These data could reflect differences in the level of HEM45, or differential accessibility of this protein to antibodies at different stages of the cell cycle. To address this issue, the amount of HEM45 protein in cell lysates of mitotic and interphase cells was examined by western immunoblot analysis (Fig.8). Mitotic cells from a synchronized cell culture produced three-fold more HEM45 protein than interphase cells.

Cytological examination of the subcellular distribution of HEM45 showed that

compartmentation of HEM45 changed during the cell cycle (Fig.4). In interphase, HEM45 staining was found as fine speckles distributed randomly throughout the cytoplasm. No nuclear staining was observed. As HeLa cells entered mitosis (in prophase), HEM45 staining became concentrated at the cytoplasmic side of the nuclear envelope. With the breakdown of the nuclear envelope, HEM45 antibody staining was amorphous but outlined the mitotic spindle fibers and the spindle caps. HEM45 staining was again found randomly distributed in the cytoplasm of telophase cells as mitosis progressed and was also observed in the midbody. At no stage of the cell cycle was HEM staining of chromosomes observed. On the contrary, chromosomes were visible by fluorescence microscopy due to their negative HEM45 staining.

In contrast, Gongora et al. (1997) report that ISG20 protein, the HEM45 equivalent, is a nuclear protein. In their study, the protein's subcellular localization was examined in CCL13, CCL39, and COS-7m6 cells following transfection with ISG20. The ISG20 gene product was observed as a nuclear protein closely associated with promyelocytic leukemia protein nuclear bodies.

Since our data suggested the possible association of HEM45 and microtubules, cells were stained with antibodies to both HEM45 and tubulin. In interphase cells, microtubules and HEM45 did not co-localize (Fig.5.A), but appeared to co-localize on spindle fibers during mitosis (Fig.5.D). Disrupting the microtubules with nocodazole had little effect on the subcellular distribution in interphase cells, but caused HEM45 to be randomly redistributed in mitotic cells. These data support the notion that HEM45 becomes microtubule associated only during mitosis, and thus is not a typical microtubule-associated-protein.

Disruption of microtubules by cold treatment caused approximately 30-40% of HEM45 staining associated with telophase cells to localize at the midbody furrow (Fig.7.B). This observation further supports the idea that HEM45 is spindle associated, since any spindle associated proteins would

likely be found in the midbody of telophase cells following cold treatment.

The biochemical basis for the association of HEM45 with spindle microtubules during metaphase and anaphase is unknown. It is plausible the cell cycle dependent association observed here between HEM45 and spindle microtubules was the result of: (i) the mitotic expression of a cellular component that binds HEM45 to spindle microtubules or (ii) HEM45 undergoing post-translational modifications during mitosis which increased the protein's affinity for spindle microtubules or another component of the mitotic apparatus. An example of this is oncoprotein 18 (op18)/ stathmin, a protein that increases the catastrophe rate of microtubules and the establishment of mitotic spindle fibers (Belmont, 1996; Sobel, 1991). Its cellular distribution is regulated by phosphorylation /dephosphorylation events and by interactions with other cellular proteins (Larsson et al., 1995). In vitro studies show that op18/stathmin is a substrate for MAP kinase and cyclin kinase p34 cdc2 (Marklund et al., 1994; Beretta et al., 1993; Luo,1994).

Fission yeast deficient in both Mik1 and Wee1 kinases enter mitosis before completion of DNA synthesis and display a mitotic catastrophe phenotype. A similar phenotype is displayed by cells missing op18/ stathmin protein or by missing phosphorylated forms of op 18/ stathmin (i.e. condensed chromosomes that appear aggregated instead of aligned at the metaphase plate) (Marklund et al., 1994; Larsson et al., 1995; Luo et al., 1994). The Xenopus XPMC2 gene product, to which HEM45 displays homology, can rescue Wee1, Mik1 yeast mutants from mitotic catastrophe (Su and Maller,1995). Su and Maller (1995) suggest that the XPMC2 gene product acts as a negative cell regulator by acting as a substrate for the cyclin kinase cdc2, like op18/stathmin. This suggests that HEM45 cellular distribution during the cell cycle may be dependent on phosphorylation. It is unknown whether HEM45 is phosphorylated in vivo, but based upon its amino acid sequence, it has a number of potential phosphorylation sites (Gongora et al., 1997).

Why the expression and subcellular localization of HEM45 change during the cell cycle is unknown. However, our observations may provide important insight into the possible biological role of HEM45. Typically, a protein's primary structure and subcellular location help predict its physiological function. At mitosis, regulation of microtubule dynamics (Mitchison and Krischner,1984) and the steady-state length of microtubules are known to be under the control of the cyclin dependent kinase activities (Verde et al.,1990). While purely speculative, it is tempting to suggest that HEM45 protein interacts with complexes on spindle fibers to influence spindle fiber dynamics. This interaction may act as a mitotic checkpoint controlling progression through mitosis. HEM45 possesses a motif commonly associated with the 3'-5' exodeoxyribonuclease (proofreading) domain of DNA polymerases, such as exonuclease REC1 (Moser et al., 1997), which couples DNA repair to a mitotic checkpoint (Onel et al., 1996). To avoid the transmission of defective chromosomes, HEM45 may act as a microtubule -nucleic acid interface protein (Moser et al., 1997) that is capable of influencing spindle fiber dynamics to delay cell mitosis if chromosomal integrity is compromised. Thus a potentially lethal attempt at the segregation of defective chromosomes (see Hartwell and Weinhart, 1989) is prevented.

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FIGURES

Figure 1. Characterization of rec HEM45 by immunoblotting using an antipeptide antiserum X826 against HEM45 peptide sequence 3-16. Silver stained gel (A) and Western immunoblot (B) of partially purified recombinant HEM45. Five μg of rec HEM45 was digested with 1.5 U of thrombin for 30 minutes and electrophoresed into a 15% SDS-polyacrylamide gel under reducing conditions. Half the gel was transferred to Immobilon P membrane and challenged with antiserum X826 (1:500 dilution). The secondary antibody was donkey anti-rabbit IgG-alkaline phosphatase (1:2,000 dilution). The blot was developed with Western Blue reagent (Promega, Madison,WI). Lanes:1. uncleaved HEM45; 2. thrombin-cleaved HEM45. The relative migration positions and size (x 1,000 daltons) of molecular weight markers are noted at the right of panel. Arrows mark the electrophoric migration position of recombinant HEM45 before and after thrombin digestion.

Figure 2. Detection of native HEM45 by western immunoblot analysis of a HeLa cell lysate. Lysates prepared from a HeLa cell culture ($2x10^5$ cells/lane) were electrophoresed into a 15% SDS-polyacrylamide gel under reducing conditions, transferred to an Immobilon P membrane, and challenged with either antipeptide antiserum X826 (A)(1:500 dilution) or rabbit Y29 antiserum (B) (1:500 dilution), prepared by immunizing rabbits with gel purified recombinant HEM45. The normal rabbit serum control for antiserum X826 produced background staining similar to that seen with preimmune serum from rabbit Y29 (see lightly stained non-specific bands in Figure 2B). Therefore the dark stained non-specific bands seen in Figure 2A were considered to be due to the ovalbumin coupled to the peptide. These could be competed out with 10mg/ml ovalbumin. The secondary antibody was donkey anti-rabbit IgG-horse radish peroxidase (1:20,000 dilution). The blot was processed for ECL detection (Amersham). The relative migration positions of molecular weight markers are noted at the right of the panel (x 1,000 daltons).

Figure 3. Immunolocalization of native HEM45 in confluent HeLa cells. Hela cells were stained with: (A) X826; (B) Y29; (C) X826 pre-incubated with immunizing peptide (10μg/ml); (D) pre-immune rabbit serum. (E) Y29 antibody; (F) phase microscopy (E). Mitotic cells in (E) are shown with white arrows. Cells were stained with primary antibody (1:500) for 1 h at RT, followed by goat anti-rabbit IgG- FITC (1:1,000 dil) for 1h at RT, and photographed at 100x magnification (A-D) or 20x magnification (E,F).

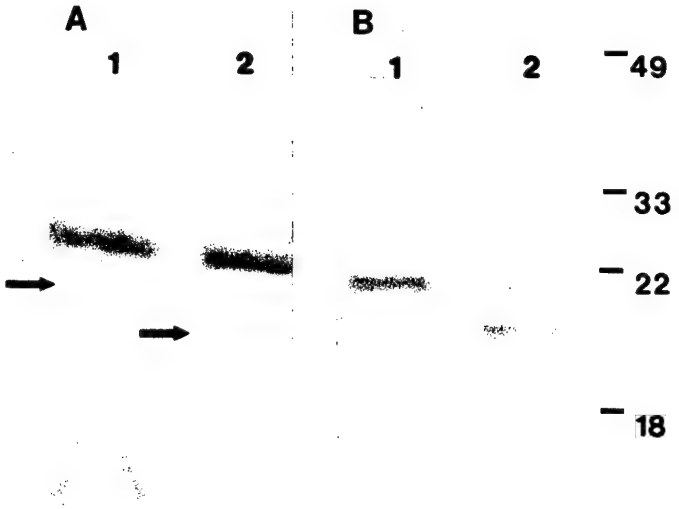
Figure 4. Localization of HEM45 in synchronized cultures of HeLa cells. Cells at various stages of the cell cycle were stained with Y29: (A) Interphase; (B) Prophase; (C) Metaphase; (D) Anaphase; (E) Telophase. Cells were stained with primary antibody (1:500) for 1 h at RT, followed by goat anti-rabbit IgG- FITC (1:1,000 dil) for 1h at RT and photographed at 200x magnification. Accompanying phase contrast micrographs are on the right (a-e).

Figure 5. Comparison of HEM45 and tubulin location in HeLa cells. Cells were stained with Y29 (A,D) or mouse anti-tubulin antibodies (B,E). Interphase cell:Y29 (A); tubulin monoclonal antibody (B); phase contrast microscopy (C). Metaphase cell:Y29 (D); tubulin antibody (E); phase contrast microscopy (F). Cells were stained with primary antibody (1:500) for 1 h at RT, followed by the appropriate secondary goat anti-rabbit IgG- FITC (1:1,000 dil) or donkey anti-mouse IgG- TRITC (1:1,000 dil) for 1 h at RT. Cells were then restained with the second primary antibody and appropriate secondary antibody and photographed at 100x magnification.

Figure 6. Nocodazole-treated HeLa interphase cells stained with Y29 (A); phase contrast microscopy (C). Nocodazole treated mitotic cell stained with antibody Y29 shown with arrow (B); Phase contrast microscopy (D). Cells were stained with primary antibody (1:500) for 1 h at RT, followed by goat anti-rabbit IgG- FITC (1:1,000 dil) for 1h at RT and photographed at 100x magnification..

Figure 7. Disruption of microtubules by cold treatment. Cells were incubated on ice for 30 min, then fixed and stained. (A) Phase contrast micrograph; (B) anti-HEM45 antibody Y29; (C) tubulin monoclonal antibody. Cells were stained with individual primary antibody (1:500) for 1 h at RT, followed the appropriate secondary goat anti-rabbit IgG- FITC (1:1,000 dil) or donkey anti-mouse IgG- TRITC (1:1,000 dil) for 1h at RT and then restained with the second primary antibody and appropriate secondary antibody and photographed at 100x magnification.

Figure 8. Comparison of HEM45 expression in interphase and mitotic HeLa cells by western blot. Mitotic cells were harvested from a cell culture 10 h after the release of a double thymidine block, while interphase cells were harvested from a nonsynchronized proliferating cell culture. Lysates ($2x10^5$ cells/lane) were electrophoresed on a 15% SDS-polyacrylamide gel, transferred to Immobilon P membrane, and challenged with antibody X826 (1:500 dilution). The secondary antibody was donkey anti-rabbit IgG-horse radish peroxidase (1:20,000 dilution). ECL detection was used. The relative migration positions of molecular weight markers are noted at the right of the panel (x 1,000 daltons). HeLa whole cell lysate from: interphase cells (A); from mitotic cells (B).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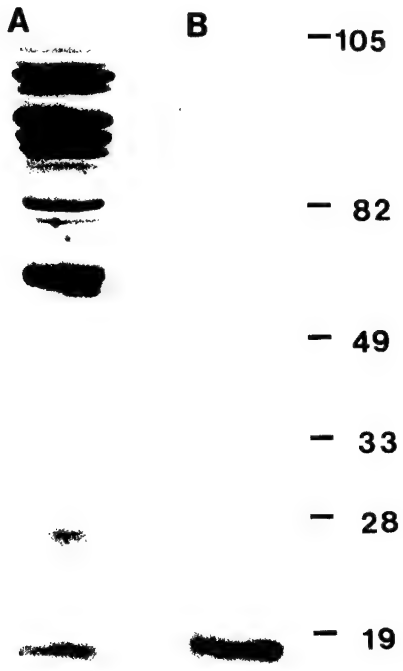
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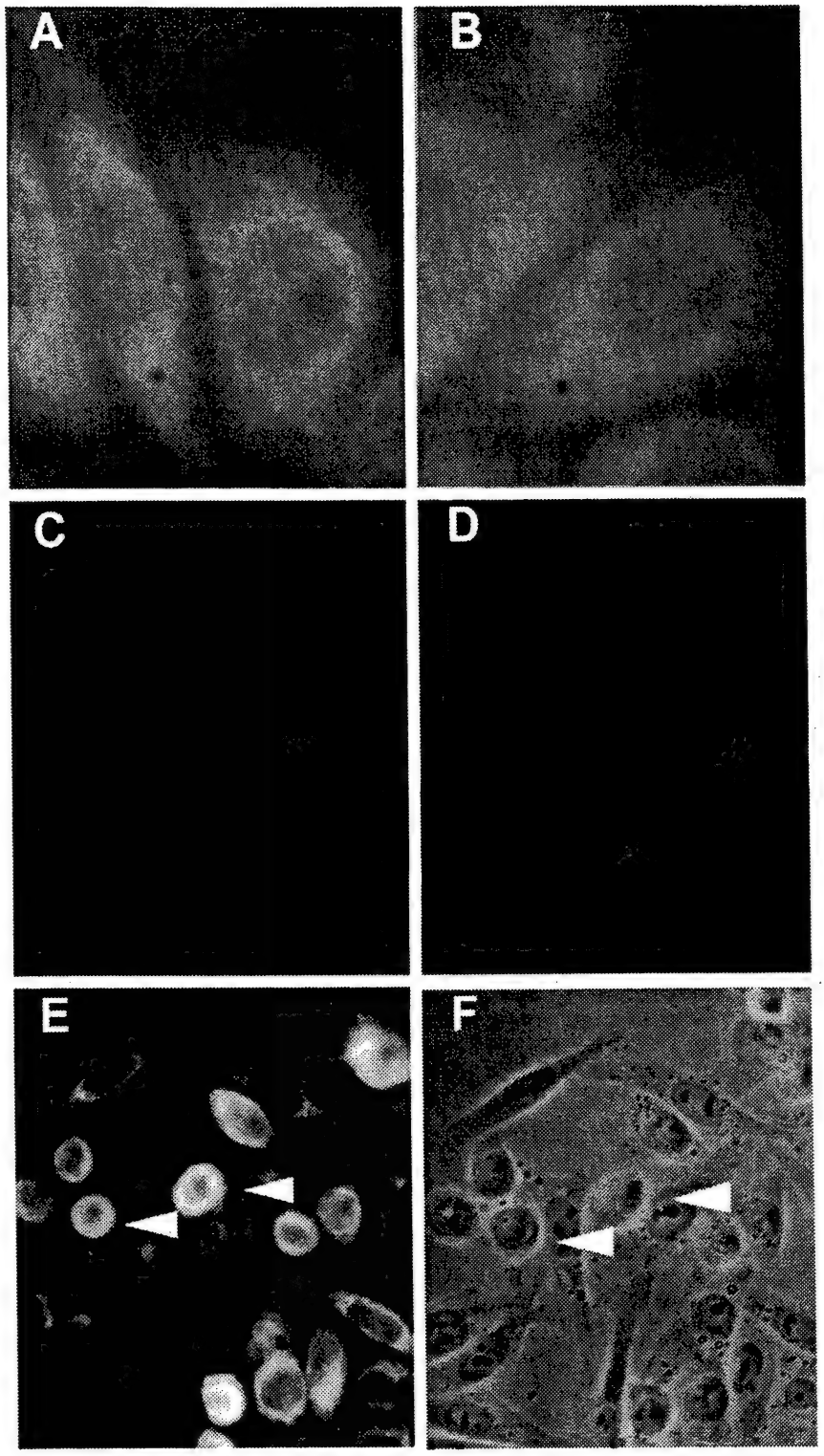
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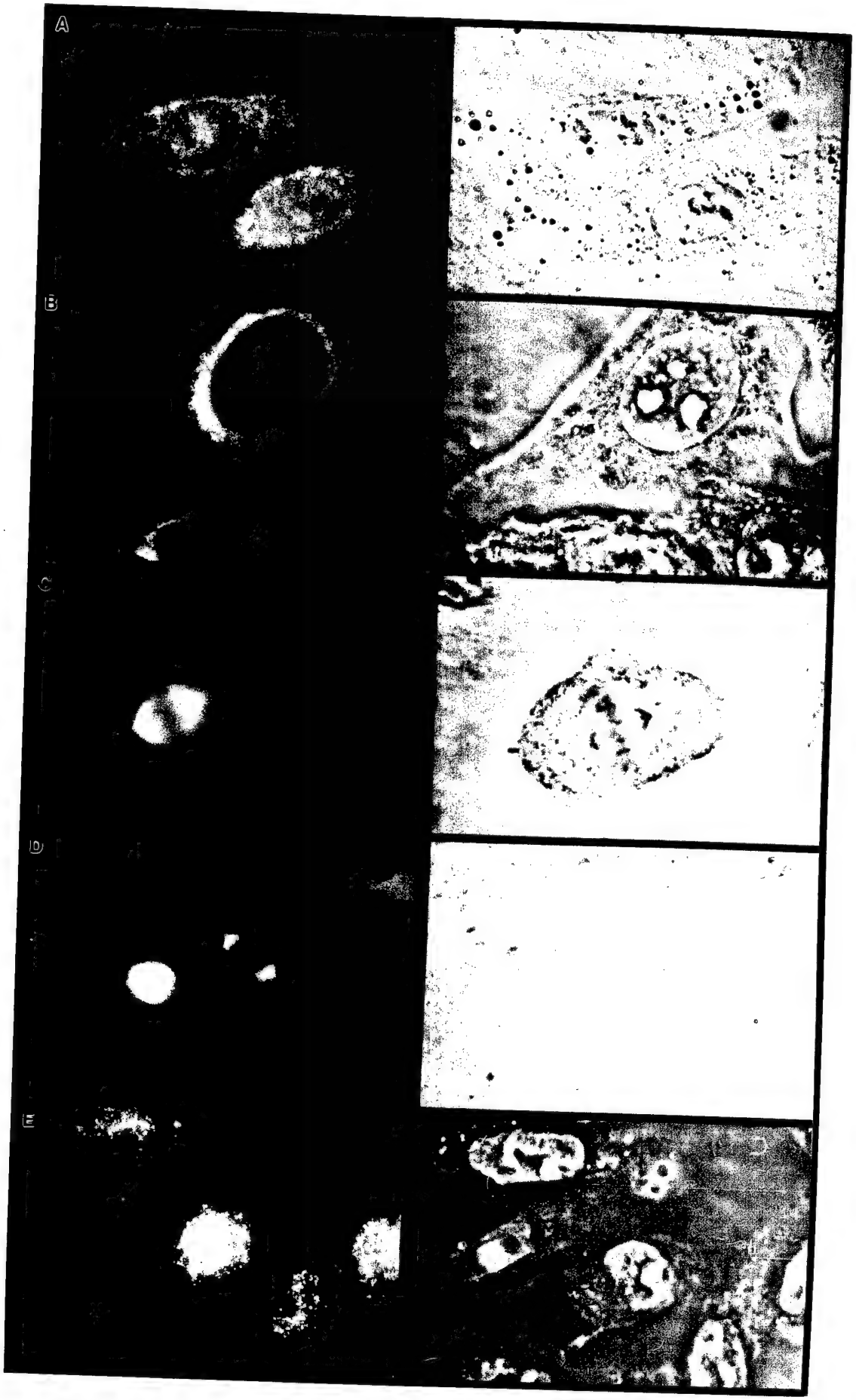
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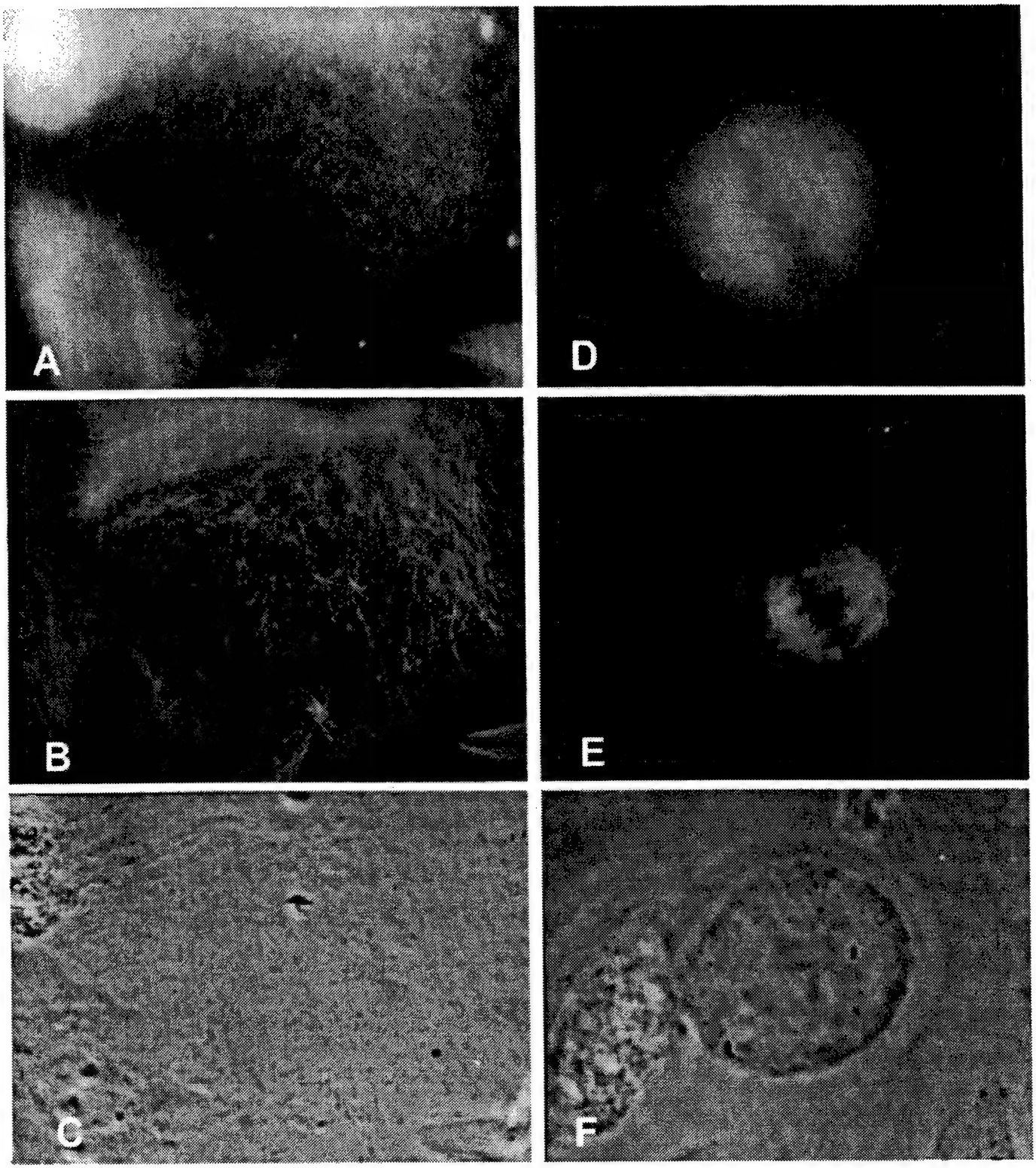
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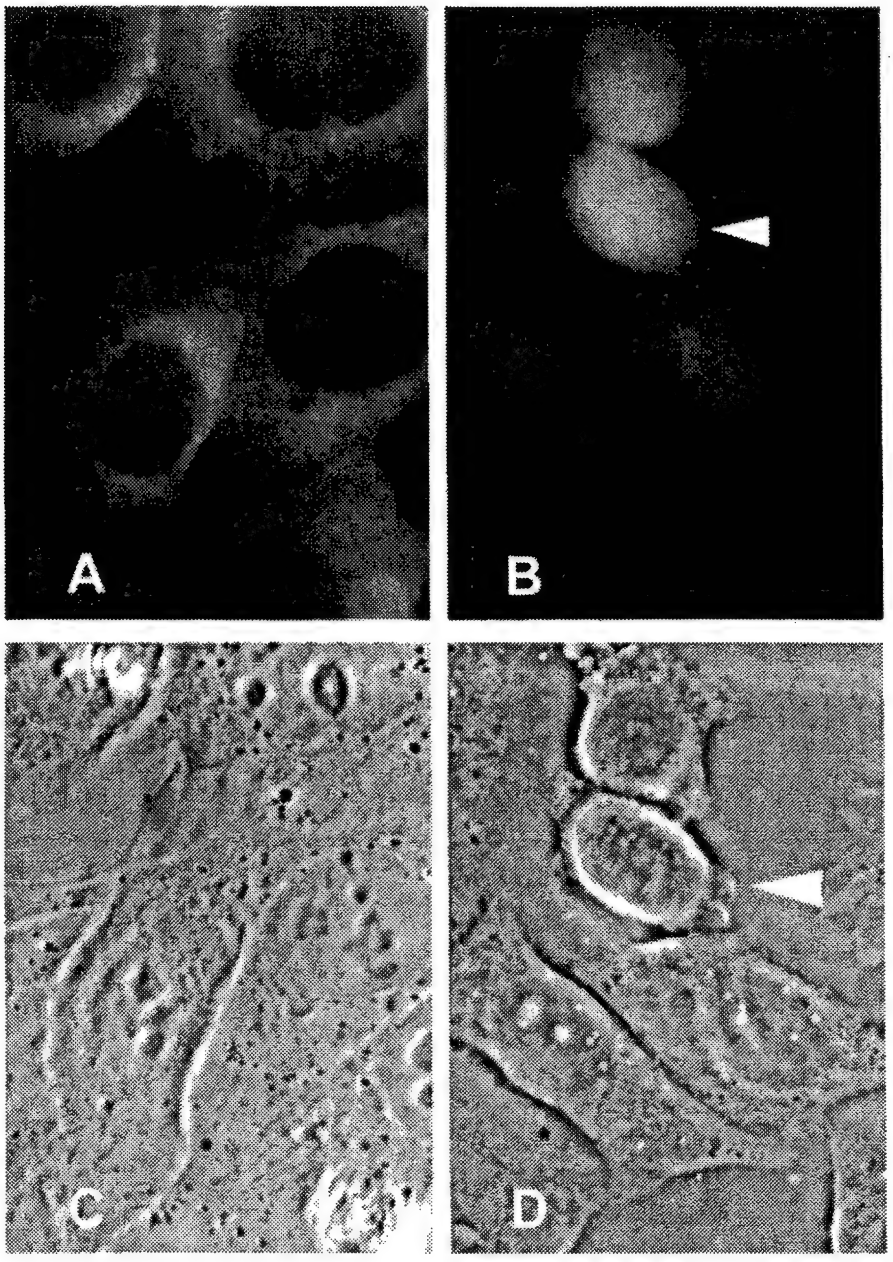
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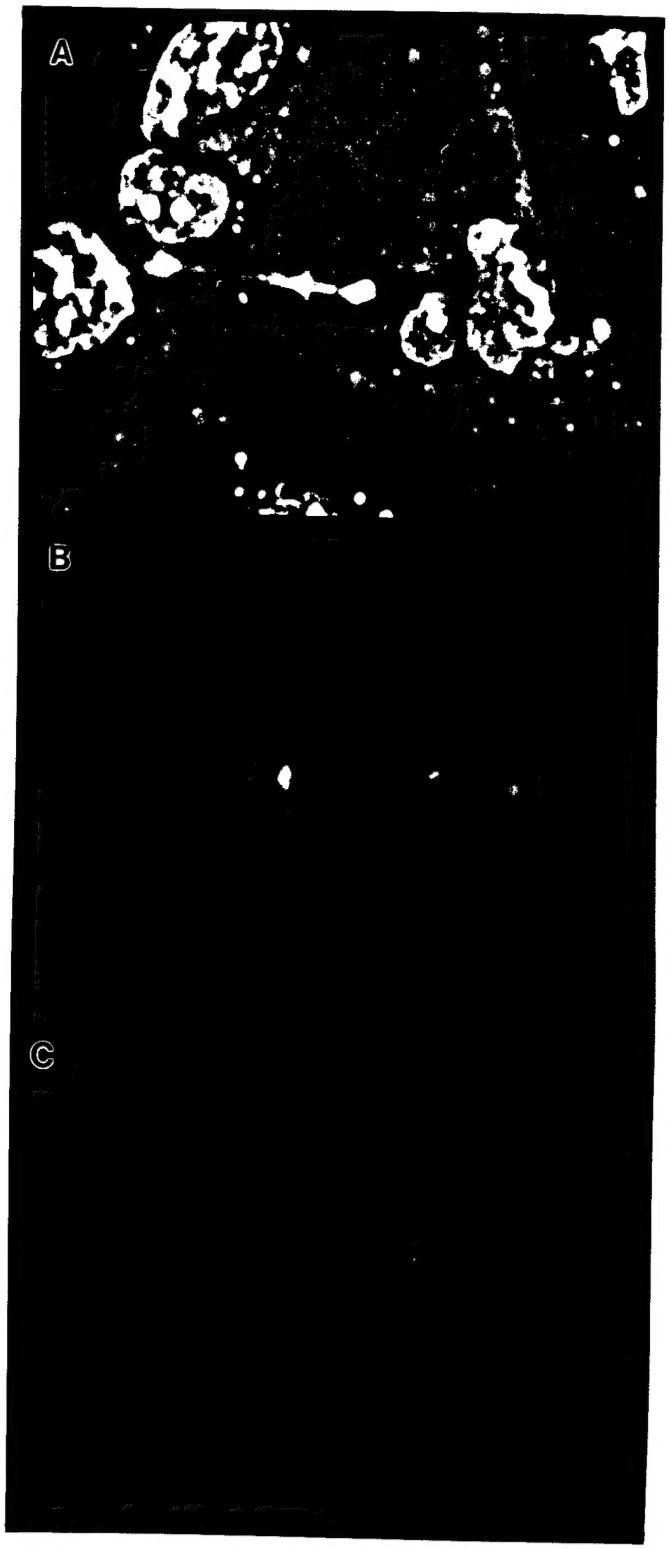
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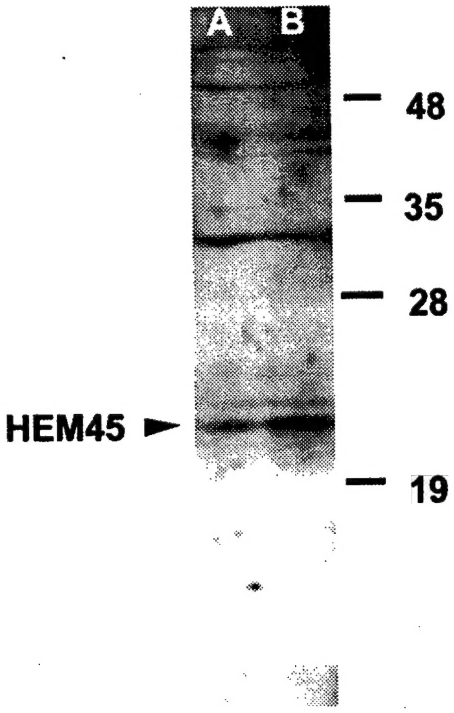
A
B
48
35
28
HEM45
19

Appendix 4: Grants applied for by PI relating to funded project

	Biology of cell cycle protein HEM45				
Title:	Pentecost				
PI:	NIH	**% Effort**	30	**$ Direct, y1:**	$156,342
Agency:	1R0101CA80952-01	**Dates:**	04/99-03/03	**$ Total:**	$987,449
Type:		**Status:**	abandoned	**Scores:**	triaged
Comments:	Study of HEM45 in cell cycle: enzyme? regulated? location? partners				

	Biology of HEM45 In Proliferating Cells				
Title:	Pentecost				
PI:	ARMY	**% Effort**	20	**$ Direct, y1:**	$70,000
Agency:	IDEA	**Dates:**	10/99-09/02	**$ Total:**	$325,827
Type:		**Status:**	not funded	**Scores:**	28th percentile
Comments:	Study of HEM45 in cell cycle: enzyme? partners? 2 AIMS OUT OF THE RO1 OF 6/1/98				

12. BINDING:

13. <u>FINAL REPORTS:</u>

Personnel supported by project dollars

Dr K Bove: Post Doc,
Alan Dupuis: technician

Publications:

Subcellular localization and expression of HEM45 in HeLa cells during the cell cycle
Peterson, AJ, Pentecost, BT and Dias JA (submitted, in revision)

Expression and estrogen regulation of the HEM45 mRNA in human tumor lines and the rat uterus
Pentecost BT J Steroid Biochemistry and Molecular Biology **64**, 25-33 (1998)